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Synthesis and Biological Evaluation of (*E*)-4-Hydroxy-3-Methylbut-2-enyl Phosphate (HMBP) Aryloxy Triester Phosphoramidate Prdrugs as Activators of Vγ9/Vδ2 T-Cells Immune Response

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Immunotherapy, T-cell, Phosphoantigen, Prodrug, ProPAgen.

ABSTRACT: The aryloxy triester phosphoramidate prodrug approach has been used with success in drug discovery. Herein, we describe the first application of this prodrug technology to the monophosphate derivative of the phosphoantigen HMBPP and one of its analogues. Some of these prodrugs exhibited specific and potent activation of Vγ9/Vδ2 T-cells, which were then able to lyse bladder cancer cells in vitro. This work highlights the promise of this prodrug technology in the discovery of novel immunotherapeutics.

INTRODUCTION

Present since birth,[1] Vγ9/Vδ2 T-cells represent the dominant subtype of human γδ T-cells in adult peripheral blood.[2] They expand in response to various infections, including tuberculosis, leprosy, typhoid, malaria, and toxoplasmosis, and studies in primate models have suggested a role in immunity to *Mycobacterium tuberculosis*.[3] Interestingly, they also exhibited an ability to target and lyse a diverse range of cancer cells in vitro.[2] Such properties have made the Vγ9/Vδ2 subset a major focus in the therapeutic exploitation of γδ T-cells.[4]

Interestingly, Vγ9/Vδ2 T-cells have been shown to be activated by small molecule phosphoantigens (PAg) such as (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) and isopentenyl pyrophosphate (IPP) (Figure 1).[5, 6] Beyond these natural ligands, two synthetic molecules, Risedronate and Zoledronate, activate Vγ9/Vδ2 T-cells through accumulation of IPP and are currently used in the clinic to treat osteoporosis and some types of cancer (Figure 1).[7-9]

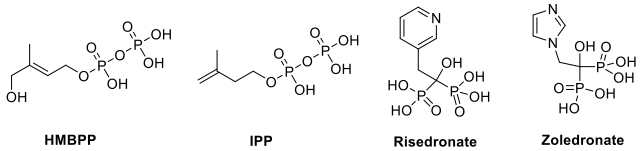


Figure 1. Chemical structures of natural phosphoantigens HMBPP and IPP as well as synthetic molecules Risedronate and Zoledronate, which activate Vγ9/Vδ2 T-cells.

The mechanism by which these small molecule phosphoantigens activate Vγ9/Vδ2 T-cells is understood to be mediated by the type-1 transmembrane protein butyrophilin 3A1.[10, 11] Although conflicting reports exist as to whether PAgs bind to the extracellular or intracellular domains of this transmembrane protein, there is an increasing body of evidence that supports the notion that these PAgs bind the intracellular B30.2 domain of butyrophilin 3A1.[10, 12-15]

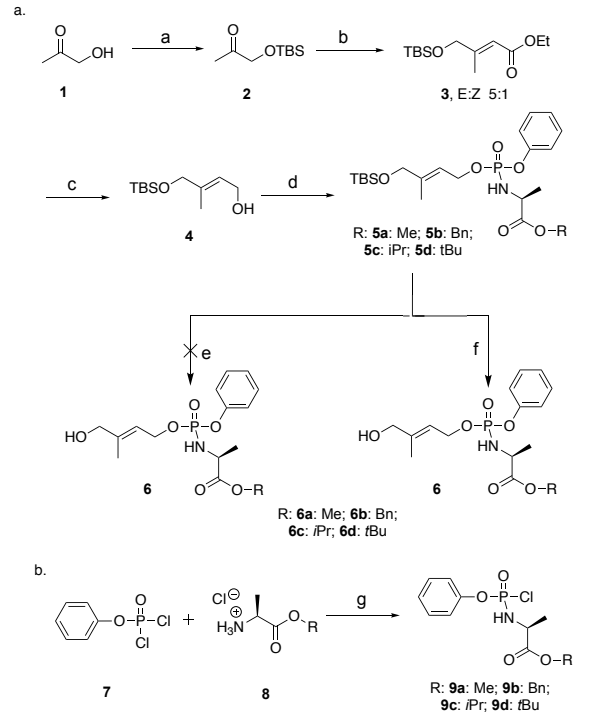
Encouraged by Vγ9/Vδ2 T-cells' ability to mount immune responses towards pathogens, lyse tumor cells, as well as their amenability to be targeted and modulated by small molecules (PAgs and their synthetic mimics), we embarked on the discovery of small molecules that have the potential to activate Vγ9/Vδ2 T-cells. Given our interest in the discovery and development of phosphorylated molecules and their prodrugs as therapeutics,[16-18] we focused our efforts on the natural phosphoantigen HMBPP as it is the most potent activator of Vγ9/Vδ2 T-cells reported to date (Figure 1).[19] HMBPP has a pyrophosphate group, which accounts for poor drug-like properties namely poor cell membrane permeability, due its charged nature under physiological conditions (pH ≤ 7.4) and limited in vivo stability. These properties have hindered the development of many drugs with unmasked phosphate or pyrophosphate groups. To overcome these drawbacks, numerous phosphate prodrug strategies have been developed and used with success in the discovery of mostly nucleotide monophosphates and monophosphonates.[20-22]

There have been reports in the literature of the application of the bis-pivaloyloxymethyl (bisPOM) phosphate prodrug technology and its derivatives to HMBP and its diphosphonates, which resulted in potent phosphoantigens though these were less potent than the parent phosphoantigen HMBPP.[19, 23-25] Aiming to discover phosphoantigen prodrugs that are as potent as HMBPP, we focused our work on the aryloxy triester phosphoramidate[26] prodrug approach, in which the monophosphate or monophosphonate groups are masked by an aryl motif and an amino acid ester moiety. This prodrug technology is known to be more efficient in delivering monophosphorylated molecules

than the bisPOM approach.[27] Over the last decade or so, it has led to at least ten clinical candidates with two being eventually approved for clinical use.[28] Notably, this prodrug approach has mostly been used on nucleotide monophosphates and monophosphonates. In this work, we applied this powerful phosphate prodrug technology to the monophosphate derivative of the phosphoantigen HMBPP, (*E*)-4-hydroxy-3-methylbut-2-enyl phosphate (HMBP). The aryloxy triester phosphoramidate prodrugs of the phosphoantigen HMBP will be referred to as HMBP ProPAgens in this work.

RESULTS AND DISCUSSION

The synthesis of HMBP ProPAgens was similar to that reported by Reichenberg et al.[29] Briefly, the hydroxyl group of 1-hydroxypropan-2-one (**1**) was protected with TBS in the presence of imidazole (Scheme 1a). The ether product **2** was then treated with a Horner-Wadsworth-Emmons reagent, specifically triethyl phosphonoacetate, to create the double bond with *E* selectivity and yield the ester product **3**. This compound, **3**, was produced in a mixture of *E*/*Z* isomers with a 5:1 ratio similar to what is reported in the literature.[5] The E/Z isomers were separated by column chromatography using solvent mixture of 5 % to 7 % diethyl ether in hexane. The two products were then evaluated with ^{1}H and ^{13}C NMR utilizing previous work by Hintz *et al*[5] who performed NOSEY experiments to determine the identity of each isomer. The desired *E*-isomer, **3**, was subsequently reduced using lithium aluminum hydride in THF to afford the key intermediate **4**. The coupling of compound **4** to phosphorochloridates to afford the desired prodrugs was achieved using standard procedures.[17, 18] Phosphorochloridates,



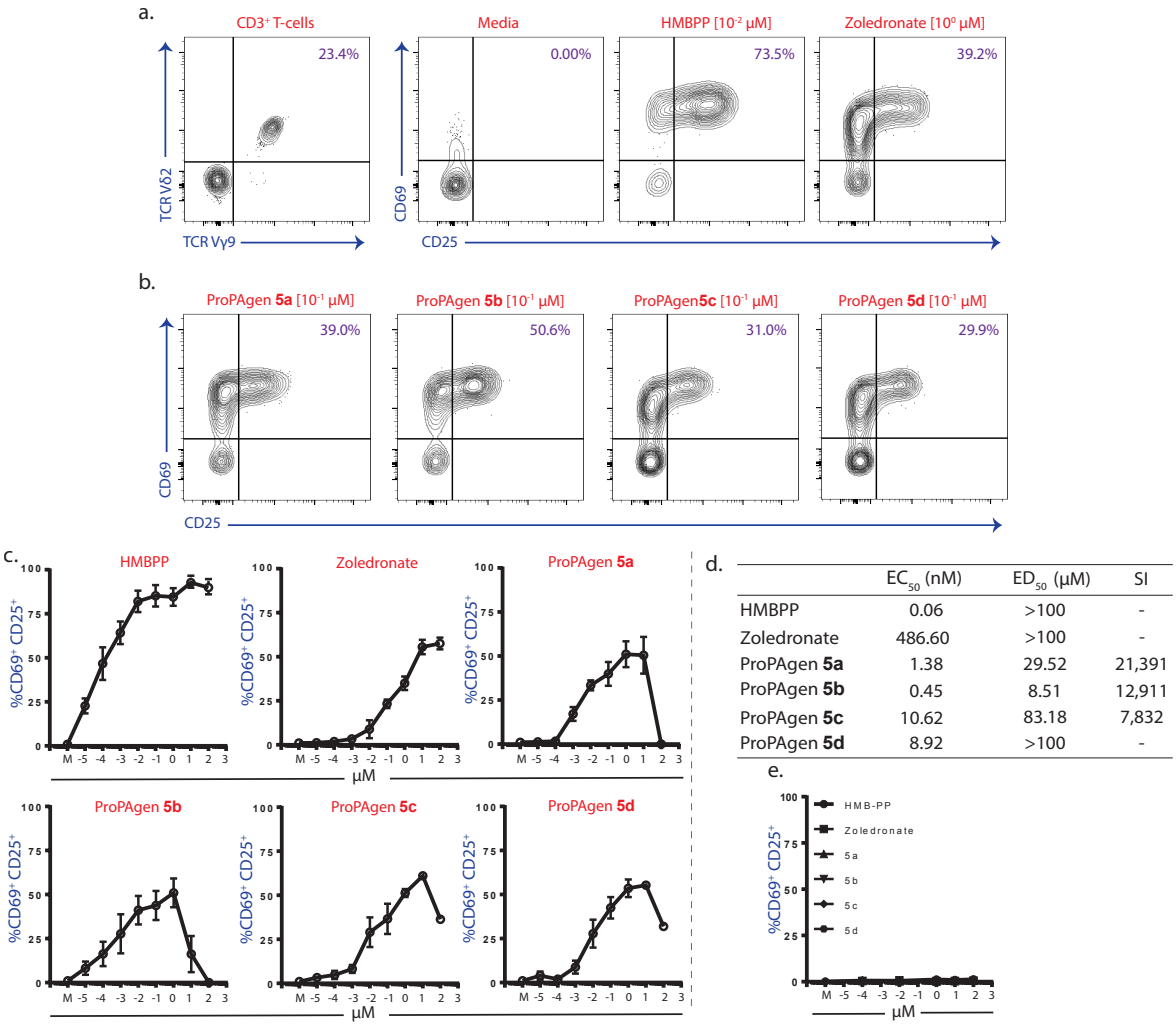
Scheme 1. A. Synthesis of HMBP ProPAgens. Reagents and conditions: (**a**) TBSCl, imidazole, DCM, rt, yield 95%; (**b**) triethyl phosphonoacetate, NaH, THF, 0 °C, yield 50%; (**c**) $LiAlH_4$, THF, 0 °C, yield 40%; (**d**) **9a**-**d**, TEA or NMI, DCM, yields 36-56%; (**e**) TBAF, THF; (**f**) HCl, MeOH, yields 20-74%. B. Synthesis of aryl phosphorochloridates. Reagents and conditions: (**g**) Et_2O, TEA, -78 °C, yields 54-95%. Me: methyl; iPr: isopropyl; tBu: tert-butyl; Bn: benzyl.

9a-d, bearing methyl, isopropyl, *tert*-butyl or benzyl esters, were synthesized following the procedure reported by Mehellou *et al*[17] and as shown in Scheme 1b. These were coupled to **4** in the presence of triethylamine or *N*-methylimidazole (NMI) to afford the desired HMBP ProPAgens, **5a-d**, in modest yields. Notably, NMI was only used in cases when TEA gave very low yields. The removal of the TBS protecting group was initially pursued using TBAF in THF.[30] However, this strategy did not work in our hands as the reaction yielded so many products and no traces of the desired HMBP ProPAgens were detected by mass spectroscopy. As an alternative, we used mild acidic conditions, 0.1 equivalents of 1.25 M HCl in methanol, and this achieved the TBS deprotection without degrading the phosphate masking moieties to afford the desired HMBP ProPAgens **6a-d** in good yields.

The unprotected HMBP ProPAgens **6a-d**, however, exhibited low stability and underwent rapid degradation, which prevented their biological testing (purity < 95%). In order to get an insight into the metabolism of ProPAgens **6a-d,** compound **6d** was incubated with the carboxypeptidase cathepsin A in vitro and the reaction was monitored with ^{31}P-NMR. The data show that after 72 h, the major metabolite had a phosphorous peak at ~ 1.95 ppm (Supporting Figure S1). Mass spectroscopy analysis of these samples showed that the degradation product, which had a ^{31}P-NMR peak of ~ 1.95 ppm, was the phosphate group masked with the aryl motif and the amino acid ester moiety (Supporting Figure S2). This indicated that the P-O bond in HMBP ProPAgens **6a-d** was liable and was cleaved off to release the unphopshorylated PAg backbone rather than HMBP. This may explain literature reports pursuing the phosphonates of HMBP where the –O-P- bond was replaced by a $–CH_2$-P- one and lack of reports on the native HMBP phosphate prodrugs.[19]

Interestingly, HMBP ProPAgens **5a-d**, which had the side chain hydroxyl group protected with a TBS moiety exhibited better stability than compounds **6a-d** and hence we were able to characterize them fully and obtain a measure of their purity (see Supporting Information). Prodrugs **5a-d** were then investigated for their ability to activate Vγ9/Vδ2 T-cells. For this, peripheral blood mononuclear cells (PBMCs) containing Vγ9/Vδ2 T-cells derived from healthy donors were incubated with increasing concentrations of HMBPP, Zoledronate or HMBP ProPAgens **5a-d** (up to 100 μM) (Figure 2a and b). Peripheral blood γδ T-cells lack appreciable levels of surface CD69 or CD25 under steady state conditions, but T-cell receptor (TCR) stimulation upregulates both T-cell activation markers.[31] PAg responsive Vγ9/Vδ2 T-cells were then distinguished by TCR Vγ9 and Vδ2 expression and assessed for the upregulation of CD69 and CD25.

As shown in Figures 2c and d, the natural phosphoantigen HMBPP showed significant activation of Vγ9/Vδ2 T-cells, EC_{50} = 0.06 nM, comparable to its reported potency.[19] Additionally, Zoledronate showed a moderate activation, EC_{50} ~ 500 nM as expected (Figures 2c and d). The four HMBP ProPAgens **5a-d** exhibited potent Vγ9/Vδ2 T-cell activation with **5b** being the most potent, EC_{50} = 0.45 nM, followed by **5a**, EC_{50} = 1.38 nM. Such potency makes HMBP ProPAgen **5b** 1,081-times more active than the clinically used agent Zoledronate. The other two HMBP ProPAgens also exhibited low nanomolar activation of Vγ9/Vδ2 T-cells, 10.62 nM for **5c** and 8.92 nM for **5d** (Figures 2c and d). Impressively, CD8+ αβ T-cells, which are activated by peptides, showed no reactivity towards HMBP ProPAgens exemplifying their specificity towards Vγ9/Vδ2 T-cells (Figure 2e). Notably, the unphopshorylated backbone



	EC_{50} (nM)	ED_{50} (µM)	SI
HMBPP	0.06	>100	-
Zoledronate	486.60	>100	-
ProPAgen **5a**	1.38	29.52	21,391
ProPAgen **5b**	0.45	8.51	12,911
ProPAgen **5c**	10.62	83.18	7,832
ProPAgen **5d**	8.92	>100	-

Figure 2. Activation of human Vγ9/Vδ2$^+$ T cells by HMBP ProPAgens. (**a**) Human peripheral blood mononuclear cells (PBMC) were incubated with media or indicated concentrations of HMB-PP or Zoledronate for 18 h. TCR Vγ9/Vδ2$^+$ T cells were then assessed for the upregulation of cell surface markers, CD69 and CD25. Data representative of n = 5. (**b**). As in (**a**), with PBMC incubated with indicated concentrations of HMBP ProPAgens. Data representative *n* = 4. (**c**). as in (**a**) and (**b**), with data showing titrations of each of HMB-PP, Zoledronate and HMBP ProPAgens, alongside a medium control (M). Data from *n* = 4-5 donors. (**d**) EC_{50}, ED_{50} and selectivity index (SI) values for each HMBP ProPAgens. Specific cell death, for ED_{50} values, was calculated after adjusting for non-specific cell death in medium controls. (**e**) CD69 and CD25 activation in CD3$^+$CD8$^+$ αβ T-cells for each compound.

compound **4** did now show any activation of Vγ9/Vδ2 T-cells (data not shown).

Next, we tested the potential for ProPAgens to sensitise the urinary bladder carcinoma cell line T24 for targeted killing by *in vitro* expanded Vγ9/Vδ2 T cells. Without sensitization, medium pulsed T24 cells were poorly targeted by increasing ratios of Vγ9/Vδ2 T cells, but upon pulsing for four hours with zoledronate or ProPAgens **5a-d**, T24 cells were specifically lysed (Figure 3a). The short pulsing period, poor lipophilicity and requirement to target a metabolic enzyme resulted in only a marginal increase in specific T24 cell killing by zoledronate (Figure 3b). In contrast, a 1000-fold lower concentration of each ProPAgen mediated a 2-4-fold increase in specific lysis of T24 over the same period (Figure 3b).

The activity across the different HMBP ProPAgens in both biological assays was in agreement with what is typically observed with aryloxy triester phosphoramidate (ProTide) prodrugs of nucleoside analogues as it correlated with the lipophilicity and rate of degradation. The HMBP ProPAgen with benzyl esters, e.g. **5b**, has higher lipophilicity and thus better (passive) cellular uptake. Also, the benzyl group is better leaving group than the other aliphatic esters and thus the metabolism of the benzyl ester of **5b** proceeds faster than the other ProPAgens, **5a**, **5c** and **5d**. Together, these explain the superior activity of ProPAgen **5b**. HMBP ProPAgen **5a** also exhibited relatively potent activation of Vγ9/Vδ2 T-cells with EC_{50} = 1.38 nM. The fact that ProPAgen **5c**, which has an isopropyl ester, exhibited lower potency in activating Vγ9/Vδ2 T-cells as compared to **5a** and **5d** was surprising as often phosphoramidate prodrugs with this ester exhibit similar activity to those with a methyl ester. However, the less potent activity observed with the HMBP ProPAgen **5d** was expected and is in agreement with the literature where phosphoramidate prodrugs with *t*Bu esters show lower biological activity than those with other ester motifs. This is because the hydrolysis of the *t*Bu ester group of the phosphoramidates by esterase enzymes proceeds much slower than those with Me, iPr and Bn esters.

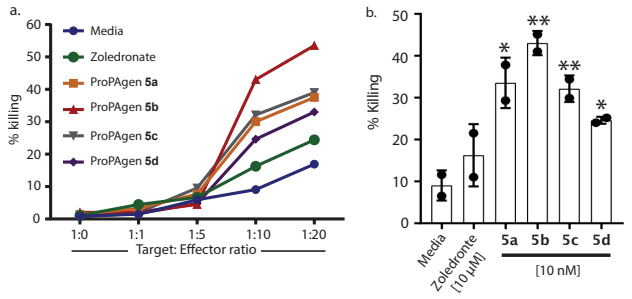


Figure 3. HMBP ProPAgens mediate the specific lysis of bladder cancer cells by Vγ9/Vδ2 T-cells. (**a**) Human T24 urinary bladder carcinoma cell lines (Target) were pulsed for 4 hours with 10 nM of the indicated ProPAgens, media or Zoledronate, then co-cultured for 18 hours with expanded Vγ9/Vδ2 T cells (Effector) at the indicated Target:Effector ratios. Specific killing of target cells was measured by an amine-reactive dead cell marker staining. Data display the mean of n = 2. (**b**). As in (**a**) showing the mean ± SD specific killing of T24 cells at 1:10 Target:Effector ratio for each indicated treatments, $n = 2$. * $P < 0.05$; ** $P < 0.01$; determined by One-way ANOVA.

CONCLUSION

We reported the application of the aryloxy triester phosphoramidate technology to the phosphoantigen HMBP and one of its synthetic analogues to generate HMBP ProPAgens. Although the ProPAgens of the native HMBP compound (**6a-d**) were of very low stability, those of the synthetic analogue (**5a-d**) were more stable. These later ones exhibited potent activation of Vγ9/Vδ2 T-cells. Notably, HMBP ProPAgen **5b** was the most potent activator of Vγ9/Vδ2 T-cells, $EC_{50} = 0.45$ nM with the other three ProPAgens, **5a**, **c** and **d**, also showing low nanomolar activation. Impressively, Vγ9/Vδ2 T-cells activated by HMBP ProPAgens exhibited potent lysis of urinary bladder carcinoma cancer cells (T24) in vitro. In terms of specificity, HMBP ProPAgens, **5a-d**, showed excellent specificity towards the activation of Vγ9/Vδ2 T-cells as they had no effects of other T-cells such as CD8+ αβ T-cells. Efforts aimed at improving the stability of the native HMBP ProPAgens are currently underway and will be reported in the future. In conclusion, the application of the aryloxy triester phosphoramidate prodrug technology to HMBP to generate HMBP ProPAgens yielded potent and specific activators of Vγ9/Vδ2 T-cells. Together, this showcases the promise of this prodrug technology in the discovery of novel immunotherapeutic agents.

EXPERIMENTAL SECTION

General Information. All of the reactions were carried out under argon atmosphere and were monitored with analytical thin layer chromatography (TLC). NMR data were recorded either on a Bruker AV300, AVIII300, AV400, AVIII400 or DRX500 spectrometers in the deuterated solvents indicated and the spectra were calibrated on residual solvent peaks. Chemical shifts (δ) are quoted in ppm and *J* values are quoted in Hz. In reporting spectral data, the following abbreviations were used: *s* (singlet), *d* (doublet), *t* (triplet), *q* (quartet), *dd* (doublet of doublets), *td* (triplet of doublets) and *m* (multiplet). HPLC was carried out on a DIONEX summit P580 quaternary low-pressure gradient pump with a built-in vacuum degasser using a Summit UVD 170s UV/Vis multichannel detector. HPLC grade solvents were used. Chromeleon software was used to visualize and process the obtained chromatograms. Analytical separations used a flow rate of 1 mL/min and preparative used a flow rate of 20 mL/min. The purity of the tested compounds was determined by high-performance liquid chromatography (HPLC) where all of the tested compounds had ≥ 95% purity.

1-((*tert*-butyldimethylsilyl)oxy)propan-2-one, 2. Hydroxyacetone **1** (1.00 g, 13.50 mmol, 1 eq.) and TBSCl (3.05 g, 20.25 mmol, 1.5 eq.) were combined in dry CH_2Cl_2 (50 mL) under an argon. Imidazole (2.02 g, 29.70, 2.2 eq.) was then added portion-wise at 0 °C. The mixture was allowed to warm to room temperature and left to stir at that temperature for 2.5 h. The reaction mixture was then washed with brine (50 mL), extracted with Et_2O (50 mL x 3), dried ($MgSO_4$) and the solvents removed under reduced pressure to give a crude oil, which was purified via column chromatography (10:1 Hexane:EtOAc). **2** obtained as a colorless oil (2.45 g, 95%). δ_H NMR (300 MHz, $CDCl_3$): 4.15 (s, 2H, CH_2), 2.17 (s, 3H, CH_3), 0.93 (s, 9H, CH_3), 0.09 (s, 6H, CH_3). δ_C NMR (101 MHz, $CDCl_3$): 209.25, 69.57, 25.98, 25.76, 18.29, -5.51. MS-ESI (%): 211.1 (100, M+Na).

Ethyl (*E*)-4-((*tert*-butyldimethylsilyl)oxy)-3-methylbut-2-enoate, 3. To a 0 °C suspension of NaH (0.20 g, 8.38 mmol, 1 eq.) in dry THF (50 mL) under an argon was added diethyl phosphonoacetate (1.88 g, 8.38 mmol, 1 eq.) dropwise. The resulting solution was left to stir for 25 min at 0 °C. Silyl ether **2** (1.5 g, 7.96 mmol, 0.95 eq.) was then added dropwise as a solution in dry THF (25 mL). The mixture was then stirred at 0 °C for a further 30 min, then warmed to room temperature and stirred for a further 2 h, or until complete via TLC. A saturated solution of brine (50 mL) was added, dropwise at first, then the organic layer was extracted with Et_2O (50 mL x 3), dried ($MgSO_4$) and the solvents were removed to give a mixture of E:Z isomers (5:1 via ^1H NMR) as a crude oil. This was purified via column chromatography (10% Et_2O in Hexane) to give product **3** as a colorless oil (1.04 g, 50%). δ_H NMR (300 MHz, $CDCl_3$): 5.99 (d, $J = 1.4$ Hz, 1H, CH), 4.17 (q, $J = 7.1$ Hz, 2H, CH_2), 4.11 (d, $J = 1.4$ Hz, 2H, CH_2), 2.05 (s (broad), 3H, CH_3), 1.29 (t, $J = 7.1$ Hz, 3H, CH_3), 0.93 (s, 9H, CH_3), 0.09 (s, 6H, CH_3). δ_C NMR (101 MHz, $CDCl_3$): 167.10, 157.16, 113.35, 67.10, 59.59, 25.88, 18.37, 15.44, 14.36, -5.45. MS-ESI (%): 258.2 (8, M), 243.2 (100, M – CH_3).

(*E*)-4-((tert-butyldimethylsilyl)oxy)-3-methylbut-2-en-1-ol, 4. In a flame dried Schleck flask were placed ester **3** (0.5 g, 1.93 mmol, 1 eq.) and 20 mL dry THF, and the resulting mixture was cooled to 0 °C with stirring. Then $LiAlH_4$ (1 M solution in THF) (1.93 mL, 1.93 mmol, 1 eq.) was added dropwise, and the solution was allowed to warm up to room temperature and stirred for 2 h. MeOH (1 mL) was then added cautiously, after which a saturated solution of sodium potassium tartrate (30 mL) was added which formed a white gel. This was stirred overnight at room temperature and then the phases were allowed to separate. The organic layer was collected and the aqueous layer was washed with Et_2O (20 mL x 3), dried ($MgSO_4$) and the solvents were removed under reduced pressure to leave a crude oil. This was then purified by column chromatography using hexane:ethyl acetate (4:1) as an eluant to give product **4** (0.1677 g, 40%) as a colorless oil. δ_H (300 MHz, $CDCl_3$) 5.72-5.65 (m, 1H, CH), 4.21 (d, $J = 6.9$ Hz, 2H, CH_2), 4.04 (s, 2H, CH_2), 1.65 (s (broad), 3H, CH_3), 0.92 (s, 9H, 3 x CH_3), 0.08 (s, 6H, CH_3). δ_C (101 MHz, $CDCl_3$) 138.32, 122.53, 67.66, 59.14, 25.94, 18.41, 13.50, -5.34. MS (ESI) (%): 239.1 (100, M+Na).

Methyl ((((*E*)-4-((*tert*-butyldimethylsilyl)oxy)-3-methylbut-2-en-1-yl)oxy)(phenoxy)phosphoryl)-*L*-alaninate, 5a. To a stirring solution of alcohol **4** (0.1301 g, 0.6166 mmol, 1 eq.) in dry CH_2Cl_2 (10 mL) was added methyl phosphorochloridate **9a** (0.2055 g, 0.7400 mmol, 1.2 eq.). The solution was cooled to 0 °C and NMI (0.10 mL g, 1.2333 mmol, 2 eq.) was added dropwise and the resulting solution was warmed to room temperature and stirred overnight. Brine (10 mL) was then added and the organic layer separated off. The aqueous layer was then extracted with Et_2O (20 mL x 3), the organic layers combined, dried ($MgSO_4$) and the solvents removed under reduced pressure to leave a crude oil. This was then

purified by column chromatography (4:1 Hex:EtOAc to 1:1 Hex:EtOAc) to give compound **5a** (0.1228 g, 43%) as a colourless oil. δ_H NMR (400 MHz, $CDCl_3$): 7.31 (t, *J* = 7.3 Hz, 2H, 2 x CH), 7.21 (t, *J* = 7.3 Hz, 2H, 2 x CH), 7.14 (t, *J* = 7.3 Hz, 1H, CH), 5.75-5.59 (m, 1H, CH), 4.72-4.64 (m, 2H, CH_2), 4.10-3.97 (m, 3H, CH_2 & CH), 3.70 (d, *J* = 8.8 Hz, 3H, CH_3), 3.53 (dd, *J* = 18.0, 9.7 Hz, 1H, NH), 1.65 (d, *J* = 3.5 Hz, 3H, CH_3), 1.37 (t, *J* = 7.4 Hz, 3H, CH_3), 0.91 (s, 9H, 3 x CH_3), 0.07 (s, 6H, 2 x CH_3). δ_C NMR (101 MHz, $CDCl_3$): 173.96 (d, *J* = 6.0 Hz), 150.89 (d, *J* = 5.7 Hz,), 141.03, 129.58, 124.70, 120.22, 117.86 (d, *J* = 7.1 Hz), 67.28, 63.28 (t, *J* = 5.0 Hz), 52.43, 50.11 (d, *J* = 4.1 Hz), 25.90, 21.05 (d, *J* = 3.3 Hz), 18.37, 13.59, -5.39. δ_P NMR (121 MHz, $CDCl_3$) 2.48, 2.50. HRMS (ESI): Found M+Na, 480.1949, [$C_{21}H_{36}NO_6SiPNa$] requires 480.1947.

Benzyl ((((*E*)-4-((*tert*-butyldimethylsilyl)oxy)-3-methylbut-2-en-1-yl)oxy)(phenoxy)phosphoryl)-*L*-alaninate, 5b. Prepared under the same procedure as described for **5a** using alcohol **4** (0.16 g, 0.77 mmol, 1 eq.), benzyl phosphorchloridate **9b** (0.32 g, 0.92 mmol, 1.2 eq.) and dry Et_3N (0.22 mL, 1.5496 mmol, 2 eq.) to give a crude yellow oil which was then purified by column chromatography (4:1 Hex:EtOAc to 2:3 Hex:EtOAc) to afford **5b** (0.1306 g, 31%) as a colourless oil. δ_H NMR (400 MHz, $CDCl_3$) δ 7.44 – 7.06 (m, 10H, Ph), 5.66 (m, 1H, CH), 5.18-5.09 (m, 2H, CH_2), 4.71-4.61 (m, 2H, CH_2), 4.13-4.04 (m, 1H, CH), 4.02 (d, *J* = 5.3 Hz, 2H, CH_2), 3.59-3.45 (m, 1H, NH), 1.63 (d, *J* = 5.1 Hz, 3H, CH_3), 1.38 (t, *J* = 6.9 Hz, 3H, CH_3), 0.91 (s, 9H, CH_3), 0.06 (s, 6H, 2 x CH_3). δ_C NMR (101 MHz, $CDCl_3$): 173.32 (2 x s), 150.88 (s), 141.06 (2 x s), 135.28, 129.59 (2 x s), 128.64 (2 x s), 128.48 (2 x s), 128.19 (2 x s), 124.71 (2 x s), 120.23 (s (broad)), 117.87 (2 x s), 67.32 (2 x s (broad)), 67.18 (2 x s (broad)), 63.35 (2 x s), 50.25 (2 x s), 25.91 (2 x s), 21.11 (2 x s), 13.59 (2 x s), -5.37. δ_P NMR (121 MHz, $CDCl_3$): 2.38, 2.56. HRMS (ESI): Found M+Na, 556.2262, [$C_{27}H_{40}NO_6PSiNa$] requires 556.2260.

Isopropyl ((((*E*)-4-((*tert*-butyldimethylsilyl)oxy)-3-methylbut-2-en-1-yl)oxy)(phenoxy)phosphoryl)-*L*-alaninate, 5c. Prepared under the same procedure as described for **5a** using alcohol **4** (0.23 g, 1.08 mmol, 1 eq.), isopropyl phosphorchloridate **9c** (0.39 g, 1.29 mmol, 1.2 eq.) and dry Et_3N (0.30 mL, 2.16 mmol, 2 eq.) to give a crude yellow oil which was then purified by column chromatography (4:1 Hex:EtOAc) to afford **5c** (0.1914 g, 36%) as a colorless oil. δ_H NMR (400 MHz, $CDCl_3$) 7.30 (td, *J* = 8.5, 2.1 Hz, 2H, 2 x CH), 7.20 (td, *J* = 7.5, 0.9 Hz, 2H, 2 x CH), 7.16-7.08 (m, 1H, CH), 5.66 (q, *J* = 7.2 Hz, 1H, CH), 5.06-4.92 (m, 1H, CH), 4.75-4.59 (m, 2H, CH_2), 4.02 (s, 2H, CH_2), 4.00-3.88 (m, 1H, CH), 3.60-3.48 (m, 1H, NH), 1.63 (s, 3H, CH_3), 1.38-1.32 (m, 3H, CH_3), 1.26-1.17 (m, 6H, 2 x CH_3), 0.90 (s, 9H, 3 x CH_3), 0.05 (s, *J* = 4.2 Hz, 6H, 2 x CH_3). δ_C NMR (101 MHz, $CDCl_3$) 173.00 (2 x d, *J* = 4.13 Hz), 150.92 (t, *J* = 6.7 Hz), 140.96, 129.57, 124.68 (s (broad)), 120.23 (t, *J* = 4.6 Hz), 117.92 (2 x s), 69.11, 67.30, 63.28 (t, *J* = 5.1 Hz), 50.30 (2 x s), 25.90, 21.66 (2 x s), 21.09 (2 x s), 18.37, 13.59, -5.39. δ_P NMR (121 MHz, $CDCl_3$): 2.62, 2.65. HRMS (ESI): Found M+Na, 508.2258, [$C_{23}H_{40}NO_6PSiNa$] requires 508.2260.

***Tert*-butyl ((((*E*)-4-((*tert*-butyldimethylsilyl)oxy)-3-methylbut-2-en-1-yl)oxy)(phenoxy)phosphoryl)-*L*-alaninate, 5d.** Prepared under the same procedure as described for **5a** using alcohol **4** (0.14 g, 0.65 mmol, 1 eq.), *tert*-butyl phosphorchloridate **9d** (0.25 g, 0.78 mmol, 1.2 eq.) and dry NMI (0.11 mL, 1.30 mmol, 2 eq.) to give a crude yellow oil which was then purified by column chromatography (4:1 Hex:EtOAc) to afford **5d** (0.1832 g, 56%) as a colorless oil. δ_H NMR (400 MHz, $CDCl_3$): 7.29 (t, *J* = 7.2 Hz, 2H, 2 x CH), 7.22 (t, *J* = 7.2, 2H, 2 x CH), 7.12 (t, *J* = 7.2 Hz, 1H, CH), 5.68 (qd, *J* = 7.2, 1.3 Hz, 1H, CH), 4.74-4.62 (m, 2H, CH_2), 4.02 (s, 2H, CH_2), 3.95-3.83 (m, 1H, CH), 3.73-3.62 (m, 1H, NH), 1.64 (s, 3H, CH_3), 1.43 (2 x s, 9H, 3 x CH_3), 1.33 (2 x d, *J* = 3.1 Hz, 3H, CH_3), 0.91 (s, 9H, 3 x CH_3), 0.07 (s, 6H, 2 x CH_3). δ_C NMR (101 MHz, $CDCl_3$): 172.64 (2 x d, *J* = 4.1 Hz), 150.94 (t, *J* = 6.2 Hz), 140.76, 129.49, 124.55, 120.21 (t, *J* = 4.8 Hz), 118.01 (2 x s (broad)), 81.69, 67.27, 63.15 (t, *J* = 5.0 Hz), 50.66 (2 x s), 27.86, 25.87, 21.06 (2 x s), 18.31, 13.53, -5.41. δ_P NMR (121 MHz, $CDCl_3$): 2.82 (s (broad)). HRMS (ESI): Found M+Na, 522.2415, [$C_{24}H_{42}NO_6SiPNa$] requires 522.2417.

Methyl ((((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)oxy)(phenoxy)phosphoryl)-*L*-alaninate, 6a. The silyl ether **5a** (0.12 g, 0.26 mmol, 1 eq.) was placed in a flask containing MeOH (3 mL). HCl (1.25 M in MeOH, 0.0215 mL, 0.0268 mmol, 0.1 eq.) was then added at 0 °C and the resulting solution was warmed to room temperature while stirring for 40 min, until complete via TLC, upon which it was neutralized with sodium bicarbonate. The mixture was then filtered, and MeOH removed under reduced pressure. Brine (10 mL) and CH_2Cl_2 (10 mL) were then added. The organic layer was collected and the aqueous layer was extracted with CH_2Cl_2 (10 mL x 3). The organic layers were combined, dried ($MgSO_4$) and the solvents removed under reduced pressure to leave a crude brown oil. This was then purified by column chromatography (4:1 Hex:EtOAc) to leave **6a** (0.0920 g, 49%) as a colorless oil. δ_H NMR (400 MHz, $CDCl_3$): 7.32 (t, *J* = 7.8 Hz, 2H, 2 x CH), 7.25-7.18 (m, 2H, 2 x CH), 7.15 (t, *J* = 7.3 Hz, 1H, CH), 5.67 (dtd, *J* = 12.5, 7.0, 1.4 Hz, 1H, CH), 4.78-4.58 (m, 2H, CH_2), 4.16-3.93 (m, 3H, CH_2 & CH), 3.71 (d, *J* = 8.4 Hz, 3H, CH_3), 3.64-3.43 (m, 1H, NH), 1.70 (s, 3H, CH_3), 1.37 (t, *J* = 7.0 Hz, 3H, CH_3). δ_C NMR (101 MHz, $CDCl_3$): 174.11 (2 x s), 150.78 (2 x s), 141.65 (s (broad)), 129.60, 124.76, 120.19 (t, J = 5.5 Hz), 118.48 (t, *J* = 7.2 Hz), 67.19, 63.49 (t, *J* = 5.7 Hz), 52.47 (s), 50.14 (2 x s), 20.95 (t, *J* = 5.3 Hz), 13.78. δ_P NMR (121 MHz, $CDCl_3$): 2.42, 2.57. HRMS (ESI): Found M+Na, 366.1081, [$C_{15}H_{22}NO_6PNa$] requires 366.1082.

Benzyl ((((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)oxy)(phenoxy)phosphoryl)-*L*-alaninate, 6b. Prepared following the same procedure as described for **6a** using silyl ether **5b** (0.13 g, 0.24 mmol, 1 eq.) and HCl (1.25 M in MeOH, 0.19 mL, 0.24 mmol, 1 eq.) in dry MeOH (5 mL), to give a crude brown oil after 2 h. This was then purified by column chromatography (1:1 Hex:EtOAc to 1:4 Hex:EtOAc) to afford **6b** (0.0207 g, 20%) as a colorless oil. δ_H NMR (400 MHz, $CDCl_3$): 7.53-7.02 (m, 10H, Ph), 5.64 (dtd, *J* = 21.5, 7.0, 1.3 Hz, 1H, CH), 5.13 (d, *J* = 11.7 Hz, 2H, CH_2), 4.75-4.57 (m, 2H, CH_2), 4.15-4.01 (m, 1H, CH), 3.99 (d, *J* = 8.9 Hz, 2H, CH_2), 3.65-3.50 (m, 1H, NH), 1.97 (s (broad), 1H, OH), 1.68 (d, *J* = 4.8 Hz, 3H, CH_3), 1.37 (t, *J* = 7.0 Hz, 3H, CH_3). δ_C NMR (100 MHz, $CDCl_3$): 173.45 (2 x d, *J* = 3.3 Hz), 150.82 (t, *J* = 6.1 Hz), 141.44 (s (broad)), 135.23, 129.60, 128.65, 128.51 (2 x s), 128.20, 124.78, 120.23 (2 x d, *J* = 5.0 Hz), 118.81 (2 x d, *J* = 6.5 Hz), 67.43, 67.24, 63.40 (t, *J* = 5.8 Hz), 50.29 (2 x s), 29.70, 13.81. δ_P NMR (121 MHz, $CDCl_3$): 2.36, 2.53. HRMS (ESI): Found M+Na, 442.1392, [$C_{21}H_{26}NO_6PNa$] requires 442.1395.

Isopropyl ((((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)oxy)(phenoxy)phosphoryl)-*L*-alaninate, 6c. Prepared following the same procedure as described for **6a** using silyl ether **5c** (0.19 g, 0.39 mmol, 1 eq.) and HCl (1.25 M in MeOH, 0.03 mL, 0.0364 mmol, 0.1 eq.) in dry MeOH (5 mL), to give a crude brown oil after 1.5 h. This was then purified by column chromatography (1:1 Hex:EtOAc) to afford **6c** (0.0725 g, 49%) as a colorless oil. δ_H NMR (400 MHz, $CDCl_3$): 7.34-7.28 (m, 2H, CH), 7.21 (t, *J* = 7.4 Hz, 2H, 2 x CH), 7.14 (t, *J* = 7.3 Hz, 1H, CH), 5.73-5.61 (m, 1H, CH), 5.08-4.91 (m, 1H, CH), 4.76-4.57 (m, 2H, CH_2), 4.04 – 3.86 (m, 3H, CH_2 & CH), 3.77-3.64 (m, 1H, NH), 2.89 (s, 1H, OH), 1.68 (s, 3H, CH_3), 1.34 (t, *J* = 6.5 Hz, 3H, CH_3), 1.22 (ddd, *J* = 11.7, 7.0, 5.5 Hz, 6H, 2 x CH_3). δ_C NMR (101 MHz, $CDCl_3$): 173.21 (2 x s), 150.90 (t, *J* = 6.2 Hz), 141.69 (s, (broad)), 129.66, 124.81, 120.28 (2 x d, *J* = 4.8 Hz), 118.63 (t, *J* = 5.7 Hz), 69.28, 67.26, 63.52 (t, *J* = 5.1 Hz), 50.37 (2 x s), 21.71 (2 x s), 21.19-20.94 (m), 13.85. δ_P NMR (121 MHz, $CDCl_3$): 2.54, 2.68.

***Tert*-butyl ((((*E*)-4-hydroxy-3-methylbut-2-en-1-yl)oxy)(phenoxy)phosphoryl)-*L*-alaninate, 6d**. Prepared following the same procedure as described for **6a** using silyl ether **5d** (0.1832 g, 0.3666

mmol, 1 eq.) and HCl (1.25 M in MeOH, 0.0293 mL, 0.0366 mmol, 0.1 eq.) in dry MeOH (3 mL), to give a crude brown oil. This was then purified by column chromatography (2:3 Hex:EtOAc) to afford **6d** (0.0983 g, 74%) as a colourless oil. δ_H NMR (400 MHz, $CDCl_3$): 7.31 (t, *J* = 7.2 Hz, 2H, 2 x CH), 7.21 (dd, *J* = 7.6, 6.6 Hz, 2H, 2 x CH), 7.13 (t, *J* = 7.3 Hz, 1H, CH), 5.73-5.61 (m, 1H, CH), 4.75-4.59 (m, 2H, CH_2), 3.99 (s (broad), 2H, CH_2), 3.96-3.80 (m, 1H, CH), 3.72-3.61 (m, 1H, NH), 2.96 (s (broad), 1H, OH), 1.68 (s, 3H, CH_3), 1.43 (2 x s, 9H, CH_3), 1.36-1.29 (t, *J* = 6.3 Hz, 3H, CH_3). δ_C NMR (101 MHz, $CDCl_3$): 172.79 (2 x s), 150.86 (t, *J* = 6.1 Hz), 141.57, 129.58, 124.69, 120.22 (2 x d, *J* = 4.9 Hz), 118.61 (2 x s), 81.97, 67.19, 63.40 (t, *J* = 5.1 Hz), 50.70, 27.89, 21.11 (2 x d, *J* = 4.6 Hz), 13.79. δ_P NMR (121 MHz, $CDCl_3$): 2.70, 2.79. HRMS (ESI): Found M+Na, 408.1554, [$C_{18}H_{28}NO_6PNa$] requires 408.1554.

Methyl (chloro(phenoxy)phosphoryl)-*L*-alaninate, 9a. Phenyl phosphorodichloridate **7** (0.2 g, 0.95 mmol, 1 eq.) and *L*-alanine methyl ester hydrogen chloride (0.13 g, 0.95 mmol, 1 eq.) were combined in dry CH_2CL_2 (10 mL) under an argon flow at -78 °C, and stirred at -78 °C for 20 minutes. Dry Et_3N (0.26 mL, 1.90 mmol, 2 eq.) was then added dropwise over 15 minutes and the reaction stirred at -78 °C for a further 30 minutes. The temperature is then raised to room temperature and the reaction stirred for a further 3.5 hours. The solvents are removed under reduced pressure, and the mixture is filtered and washed with Et_2O, which is removed under reduced pressure to give a crude oil. This is then purified by column chromatography (7:3 Hex:EtOAc) to give **9a** as a colorless oil (0.14 g, 54%), which is stored under argon. δ_H NMR (300 MHz, $CDCl_3$): 7.40-7.34 (m, 2H, 2 x CH), 7.29-7.23 (m, 3H, 2 x CH), 4.66-4.49 (m, 1H, NH), 4.28-4.12 (m, 1H, CH), 3.78 (2 x s, 3H, CH3), 1.51 (2 x dd, *J* = 4.1, 0.5 Hz, 3H, CH3). δ_C (101 MHz, $CDCl_3$): 173.11 (2 x d, *J* = 8.8 Hz), 149.73 (2 x d, *J* = 7.14 Hz), 129.90, 125.99, 120.54 (2 x d, *J* = 1.4 Hz), 52.80 (2 x s), 50.39 (2 x d, *J* = 1.5 Hz), 20.51 (2 x d, *J* = 1.5 Hz). δ_P NMR (121 MHz, $CDCl_3$): 7.62, 7.94.

Benzyl (chloro(phenoxy)phosphoryl)-*L*-alaninate, 9b. Followed same procedure as described for **9a** using **7** (0.35 mL, 3.32 mmol, 1 eq.), Et_3N (0.63 mL, 4.64 mmol, 2 eq.) and *L*-alanine benzyl ester hydrogen chloride (0.5 g, 2.32 mmol, 1 eq.) to give phosphorchloridate **9b** (0.505 g, 95%). δ_H NMR (300 MHz, $CDCl_3$): 7.42-7.32 (m, 7H, Ph), 7.25-7.20 (m, 3H, Ph), 5.21 (2 x s, 2H, CH2), 4.34-4.15 (m, 2H, CH & N-H), 1.52 (dd, *J* = 6.6, 2.4 Hz, 3H, CH3). δ_C NMR (101 MHz, $CDCl_3$): 172.52 (dd, J = 11.6, 8.7 Hz), 149.75 (dd, *J* = 11.6. 8.7 Hz), 135.05 (d, *J* = 5.8 Hz, C-10), 129.92, 128.68, 128.59, 128.33, 125.99, 120.57, 67.60, 50.67, 20.47 (t, *J* = 4.5 Hz). δ_P NMR (121 MHz, $CDCl_3$): 7.49, 7.85.

Isopropyl (chloro(phenoxy)phosphoryl)-*L*-alaninate, 9c. Followed same procedure as described for **9a** using **7** (0.62 mL, 4.18 mmol, 1 eqvs), Et_3N (1.13 mL, 8.36 mmol, 2 eqvs) and *L*-alanine isopropyl ester hydrogen chloride (0.700 g, 4.18 mmol, 1 eqvs) to give phosphorchloridate **9c** (1.185 g, 93%). δ_H NMR (300 MHz, $CDCl_3$): 7.41- 7.34 (m, 2H, 2 x CH), 7.30- 7.21 (m, 3H, 3 x CH), 5.15-5.01 (m, 1H, CH), 4.50-4.31 (m, 1H, NH), 4.22-4.04 (m, 1H, CH), 1.50 (dd, *J* = 7.0, 2.1 Hz, 3H, CH_3), 1.34-1.22 (m, 6H, 2 x CH_3). δ_C NMR (101 MHz, $CDCl_3$): 172.15 (dd, *J* = 13.9, 9.2 Hz), 149.76 (t, *J* = 7.0 Hz), 129.93, 125.98, 120.56 (d, *J* = 4.9 Hz), 69.79 (d, *J* = 10.5 Hz), 50.70 (2 x s), 21.67 (2 x s), 20.55 (d, *J* = 4.4 Hz). δ_P NMR (121 MHz, $CDCl_3$): 7.76, 8.11.

***tert*-butyl (chloro(phenoxy)phosphoryl)-*L*-alaninate, 9d.** Followed same procedure as described for **9a** using 7 (0.22 mL, 1.5 mmol, 1 eq.), Et_3N (0.42 mL, 3 mmol, 2 eq.) and *L*-alanine *tert*-butyl ester hydrogen chloride (0.27 g, 1.5 mmol, 1 eq.) to give phosphorchloridate **9d** (0.44 g, 90%). δ_H NMR (400 MHz, $CDCl_3$): 7.37 (t, *J* = 7.8 Hz, 2H, 2 x CH), 7.30-7.22 (m, 3H, 2 x CH), 4.46-4.24 (m, 1H, NH), 4.16-3.97 (m, 1H, CH), 1.50-1.46 (m, 12H, 4 x CH_3). δ_C NMR (101 MHz, $CDCl_3$): 171.77 (dd, *J* = 15.8, 9.4 Hz), 149.80, 129.92, 125.96, 120.57 (d, *J* = 5.0 Hz), 82.70 (2 x s), 51.09 (2 x s), 27.93, 20.63. δ_P NMR (121 MHz, $CDCl_3$): 7.85, 8.24.

ASSOCIATED CONTENT

Supporting information

The Supporting Information is available free of charge on the ACS Publications website at DOI: xxx.

T-cell isolation, culture and activation. (PDF)
In vitro cathepsin A mediated degradation of **6d**. (PDF)
^{1}H NMR and ^{13}C NMR spectra, HPLC data, and mass spectra. (PDF)
Molecular formula strings. (CSV)

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Author Contributions

RM and RCM synthesised all of the reported compounds. MSD, ATB and TET carried out the biological testing of the prodrugs. CSL assisted with the NMR and mass spectroscopy studies. BEW and YM designed and supervised the experiments. YM wrote the manuscript and all of the authors gave approval to the final version of the manuscript. ‡These authors contributed equally.

ABBREVIATIONS USED

Bis-POM, bis-pivaloyloxymethyl; Bn, benzyl; HMBP, (*E*)-4-hydroxy-3-methyl-but-2-enyl monophosphate; HMBPP, (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate; IPP, isopentenyl pyrophosphate; iPr, isopropyl; Me, methyl; NMI, *N*-methylimidazole; PAg, phosphoantigen; PBMC, peripheral blood mononuclear cell; ProPAgen: prodrug of a phosphoantigen; TBAF, tetrabutylammonium fluoride; TBS: *tert*-butyldimethylsilyl; *t*Bu, *tert*-butyl; TCR, T-cell receptor; THF, tetrahydrofuran.

REFERENCES

1. Dimova, T.; Brouwer, M.; Gosselin, F.; Tassignon, J.; Leo, O.; Donner, C.; Marchant, A.; Vermijlen, D. Effector Vγ9Vδ2 T cells dominate the human fetal γδ T-cell repertoire. *Proc. Natl. Acad. Sci. U. S. A.* **2015,** *112*, E556-565.
2. Morita, C. T.; Jin, C.; Sarikonda, G.; Wang, H. Nonpeptide antigens, presentation mechanisms, and immunological memory of human Vγ2Vδ2 T cells: discriminating friend from foe through the recognition of prenyl pyrophosphate antigens. *Immunol. Rev.* **2007,** *215*, 59-76.
3. Shen, Y.; Zhou, D.; Qiu, L.; Lai, X.; Simon, M.; Shen, L.; Kou, Z.; Wang, Q.; Jiang, L.; Estep, J.; Hunt, R.; Clagett, M.; Sehgal, P. K.; Li, Y.; Zeng, X.; Morita, C. T.; Brenner, M. B.; Letvin, N. L.; Chen, Z. W. Adaptive immune response of Vγ2Vδ2+ T cells during mycobacterial infections. *Science* **2002,** *295*, 2255-2258.
4. Fisher, J. P.; Heuijerjans, J.; Yan, M.; Gustafsson, K.; Anderson, J. γδ T cells for cancer immunotherapy: a systematic review of clinical trials. *Oncoimmunology* **2014,** *3*, e27572.
5. Hintz, M.; Reichenberg, A.; Altincicek, B.; Bahr, U.; Gschwind, R. M.; Kollas, A. K.; Beck, E.; Wiesner, J.; Eberl, M.; Jomaa, H. Identification of (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate as a major activator for human γδ T cells in Escherichia coli. *FEBS Lett.* **2001,** *509*, 317-322.
6. Tanaka, Y.; Morita, C. T.; Tanaka, Y.; Nieves, E.; Brenner, M. B.; Bloom, B. R. Natural and synthetic non-peptide antigens recognized by human γδ T cells. *Nature* **1995,** *375*, 155-158.

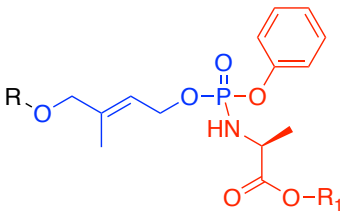
7. Maraka, S.; Kennel, K. A. Bisphosphonates for the prevention and treatment of osteoporosis. *BMJ* **2015,** *351*, h3783.
8. Neville-Webbe, H. L.; Holen, I.; Coleman, R. E. The anti-tumour activity of bisphosphonates. *Cancer Treat. Rev.* **2002,** *28*, 305-319.
9. Roelofs, A. J.; Jauhiainen, M.; Monkkonen, H.; Rogers, M. J.; Monkkonen, J.; Thompson, K. Peripheral blood monocytes are responsible for γδ T cell activation induced by zoledronic acid through accumulation of IPP/DMAPP. *Br. J. Haematol.* **2009,** *144*, 245-250.
10. Vavassori, S.; Kumar, A.; Wan, G. S.; Ramanjaneyulu, G. S.; Cavallari, M.; El Daker, S.; Beddoe, T.; Theodossis, A.; Williams, N. K.; Gostick, E.; Price, D. A.; Soudamini, D. U.; Voon, K. K.; Olivo, M.; Rossjohn, J.; Mori, L.; De Libero, G. Butyrophilin 3A1 binds phosphorylated antigens and stimulates human γδ T cells. *Nat. Immunol.* **2013,** *14*, 908-916.
11. Harly, C.; Guillaume, Y.; Nedellec, S.; Peigne, C. M.; Monkkonen, H.; Monkkonen, J.; Li, J.; Kuball, J.; Adams, E. J.; Netzer, S.; Dechanet-Merville, J.; Leger, A.; Herrmann, T.; Breathnach, R.; Olive, D.; Bonneville, M.; Scotet, E. Key implication of CD277/butyrophilin-3 (BTN3A) in cellular stress sensing by a major human γδ T-cell subset. *Blood* **2012,** *120*, 2269-2279.
12. Rhodes, D. A.; Chen, H. C.; Price, A. J.; Keeble, A. H.; Davey, M. S.; James, L. C.; Eberl, M.; Trowsdale, J. Activation of human γδ T cells by cytosolic interactions of BTN3A1 with soluble phosphoantigens and the cytoskeletal adaptor periplakin. *J. Immunol.* **2015,** *194*, 2390-2398.
13. Sandstrom, A.; Peigne, C. M.; Leger, A.; Crooks, J. E.; Konczak, F.; Gesnel, M. C.; Breathnach, R.; Bonneville, M.; Scotet, E.; Adams, E. J. The intracellular B30.2 domain of butyrophilin 3A1 binds phosphoantigens to mediate activation of human Vγ9Vδ2 T cells. *Immunity* **2014,** *40*, 490-500.
14. Wang, H.; Morita, C. T. Sensor function for butyrophilin 3A1 in prenyl pyrophosphate stimulation of human Vγ2Vδ2 T Cells. *J. Immunol.* **2015,** *195*, 4583-4594.
15. Salim, M.; Knowles, T. J.; Baker, A. T.; Davey, M. S.; Jeeves, M.; Sridhar, P.; Wilkie, J.; Willcox, C. R.; Kadri, H.; Taher, T. E.; Vantourout, P.; Hayday, A.; Mehellou, Y.; Mohammed, F.; Willcox, B. E. BTN3A1 discriminates γδ T cell phosphoantigens from nonantigenic small molecules via a conformational sensor in its B30.2 domain. *ACS Chem. Biol.* **2017,** *12*, 2631–2643.
16. Mehellou, Y.; McGuigan, C.; Brancale, A.; Balzarini, J. Design, synthesis, and anti-HIV activity of 2',3'-didehydro-2',3'-dideoxyuridine (d4U), 2',3'-dideoxyuridine (ddU) phosphoramidate 'ProTide' derivatives. *Bioorg. Med. Chem. Lett.* **2007,** *17*, 3666-3669.
17. Mehellou, Y.; Valente, R.; Mottram, H.; Walsby, E.; Mills, K. I.; Balzarini, J.; McGuigan, C. Phosphoramidates of 2'-*β*-D-arabinouridine (AraU) as phosphate prodrugs; design, synthesis, in vitro activity and metabolism. *Bioorg. Med. Chem.* **2010,** *18*, 2439-2446.
18. Osgerby, L.; Lai, Y. C.; Thornton, P. J.; Amalfitano, J.; Le Duff, C. S.; Jabeen, I.; Kadri, H.; Miccoli, A.; Tucker, J. H.; Muqit, M. M.; Mehellou, Y. Kinetin riboside and its ProTides activate the Parkinson's disease associated PTEN-induced putative kinase 1 (PINK1) independent of mitochondrial depolarization. *J. Med. Chem.* **2017,** *60*, 3518–3524.
19. Hsiao, C. H.; Lin, X.; Barney, R. J.; Shippy, R. R.; Li, J.; Vinogradova, O.; Wiemer, D. F.; Wiemer, A. J. Synthesis of a phosphoantigen prodrug that potently activates Vγ9Vδ2 T-lymphocytes. *Chem. Biol.* **2014,** *21*, 945-954.
20. Hecker, S. J.; Erion, M. D. Prodrugs of phosphates and phosphonates. *J. Med. Chem.* **2008,** *51*, 2328-2345.
21. Thornton, P. J.; Kadri, H.; Miccoli, A.; Mehellou, Y. Nucleoside phosphate and phosphonate prodrug clinical candidates. *J. Med. Chem.* **2016,** *59*, 10400-10410.
22. Pradere, U.; Garnier-Amblard, E. C.; Coats, S. J.; Amblard, F.; Schinazi, R. F. Synthesis of nucleoside phosphate and phosphonate prodrugs. *Chem. Rev.* **2014,** *114*, 9154-9218.
23. Kilcollins, A. M.; Li, J.; Hsiao, C. H.; Wiemer, A. J. HMBPP analog prodrugs bypass energy-dependent uptake to promote efficient BTN3A1-mediated malignant cell lysis by Vγ9Vδ2 T lymphocyte effectors. *J. Immunol.* **2016,** *197*, 419-428.
24. Shippy, R. R.; Lin, X.; Agabiti, S. S.; Li, J.; Zangari, B. M.; Foust, B. J.; Poe, M. M.; Hsiao, C. C.; Vinogradova, O.; Wiemer, D. F.; Wiemer, A. J. Phosphinophosphonates and their tris-pivaloyloxymethyl prodrugs reveal a negatively cooperative butyrophilin activation mechanism. *J. Med. Chem.* **2017,** *60*, 2373-2382.
25. Foust, B. J.; Poe, M. M.; Lentini, N. A.; Hsiao, C. C.; Wiemer, A. J.; Wiemer, D. F. Mixed aryl phosphonate prodrugs of a butyrophilin ligand. *ACS Med. Chem. Lett.* **2017,** *8*, 914-918.
26. Mehellou, Y.; Rattan, H. S.; Balzarini, J. The ProTide prodrug technology: from the concept to the clinic. *J. Med. Chem.* [Online early access]. DOI: 10.1021/acs.jmedchem.7b00734. Published Online: August 9, 2017.
27. Eisenberg, E. J.; He, G. X.; Lee, W. A. Metabolism of GS-7340, a novel phenyl monophosphoramidate intracellular prodrug of PMPA, in blood. *Nucleosides Nucleotides Nucleic Acids* **2001,** *20*, 1091-1098.
28. Mehellou, Y. The ProTides boom. *ChemMedChem* **2016,** *11*, 1114-1116.
29. Reichenberg, A.; Hintz, M.; Kletschek, Y.; Kuhl, T.; Haug, C.; Engel, R.; Moll, J.; Ostrovsky, D. N.; Jomaa, H.; Eberl, M. Replacing the pyrophosphate group of HMB-PP by a diphosphonate function abrogates its potential to activate human γδ T cells but does not lead to competitive antagonism. *Bioorg. Med. Chem. Lett.* **2003,** *13*, 1257-1260.
30. Corey, E. J.; Venkateswarlu, A. Protection of hydroxyl groups as *tert*-butyldimethylsilyl derivatives. *J. Am. Chem. Soc.* **1972,** *94*, 6190-6191.
31. Davey, M. S.; Lin, C. Y.; Roberts, G. W.; Heuston, S.; Brown, A. C.; Chess, J. A.; Toleman, M. A.; Gahan, C. G.; Hill, C.; Parish, T.; Williams, J. D.; Davies, S. J.; Johnson, D. W.; Topley, N.; Moser, B.; Eberl, M. Human neutrophil clearance of bacterial pathogens triggers anti-microbial γδ T cell responses in early infection. *PLoS Pathog.* **2011,** *7*, e1002040.

Table of Contents graphic:

Phosphoantigen Prodrugs (ProPAgens)

Specific activators of Vγ9/Vδ2 T-cells, EC_{50} = 0.45 to 11 nM

Potent lysis of bladder carcinoma cancer cells



One ProPAgen > 1000-times more potent than Zoledronate

SUPPORTING INFORMATION

Synthesis and Biological Evaluation of (*E*)-4-Hydroxy-3-Methylbut-2-enyl Phosphate (HMBP) Aryloxy Triester Phosphoramidate Prodrugs as Activators of Vγ9/Vδ2 T-Cells Immune Response

Martin S. Davey,[a,‡] Roshni Malde,[b,‡] Rory C. Mykura,[b,‡] Alfie T. Baker,[a] Taher E. Taher,[a] Cécile S. Le Duff,[b] Benjamin E. Willcox,[*,a] Youcef Mehellou[*,c]

[a]Cancer Immunology and Immunotherapy Centre, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK.

[b]School of Chemistry, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK.

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Table of contents:

I. T-cell isolation, culture and activation

PBMCs were isolated from heparinised venous blood from consented healthy donors (approved by the NRES Committee West Midlands ethical board; REC reference 14/WM/1254). Blood was layered over lymphoprep© (Stem Cell Technologies) and PBMC isolated according to manufacturers instructions. The cell culture medium used throughout was RPMI-1640 media supplemented with 2 mM L-glutamine, 25 mM HEPES, 1% sodium pyruvate, 50 µg/ml penicillin/streptomycin (Invitrogen) and 10% fetal calf serum (Sigma). For activation assays, 5 x 10^5 PBMC were cultured for 20 h in the presence of medium alone or the indicated concentrations of HMB-PP (Sigma), Zoledronate (Sigma), **5a**, **5b**, **5c** and **5d** (as described above). Cultured PBMC were labelled with Zombie© aqua viability dye (Biolegend) and cells were stained for surface antigens by antibodies directed against CD3 (UCHT1; 1:100), CD8 (SK1; 1:200), CD25 (2A3; 1:200); all Biolegend, CD69 (TP1.55.3; 1:20) and TCR Vγ9 (IMMU360; 1:400); Beckman Coulter, and TCR Vδ2 (123R3; 1:200); Miltenyi. For killing assays, Vγ9/Vδ2 T cells were expanded from 2 x 10^5/ml PBMC cultured with 10 µM zoledronate for 14 days and supplemented with 100 U/ml IL-2 into the media every 2-3 days, yielding 83 – 91% Vγ9/Vδ2 T cells. T24 (ATCC HTB4) cell line were labelled with 0.1µM CFSE and incubated for 4 hours with 10 µM zoledronate or 10 nM ProPAgens (**5a** - **d**), before being washed five-times in medium and co-cultured with expanded Vγ9/Vδ2 T cells for 18 hours. All cells were then labelled with Zombie© aqua viability dye and $CFSE^+$ Zombie© $aqua^+$ cells measured. All data were acquired on an LSR II (Beckton Dickinson) and data analysed with FlowJo V10.1 (TreeStar). Tabulated data were analysed in Graphpad PRISM 7 (Graphpad Software Inc).

Supporting Figure S1.

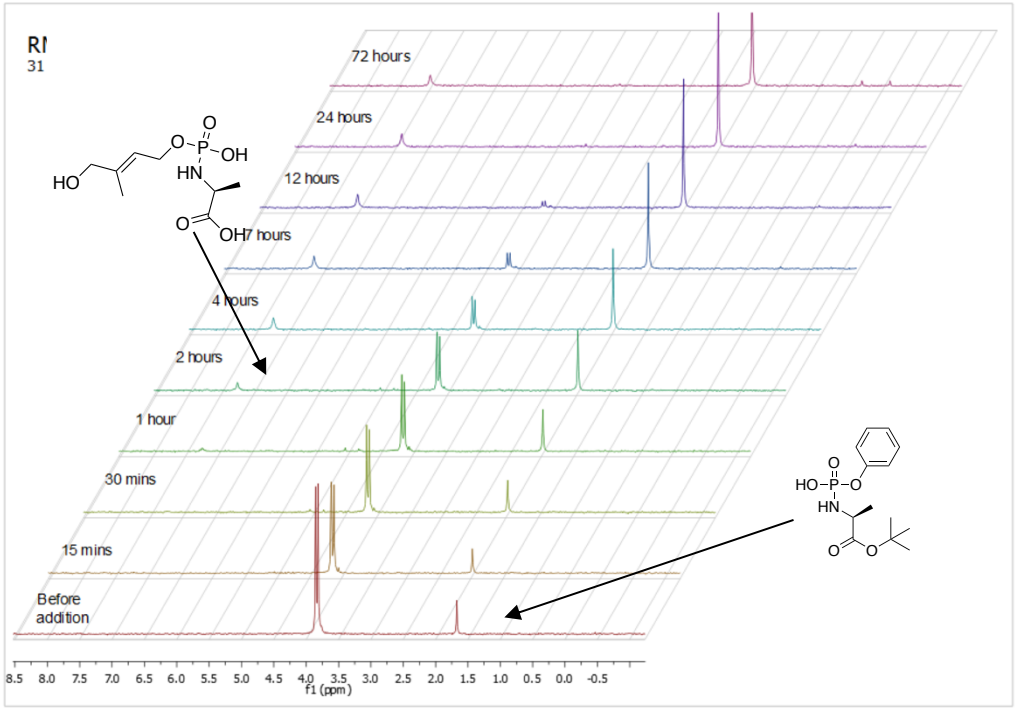


Figure S1: ^{31}P NMR of HMBP ProPAgen 6d incubated with the carboxypeptisade cathepsin A. The two singlets at 4.02 ppm corresponds to the two diastereoisomers of ProPAgen 6d. The singlet at 6.87 ppm corresponds to the amino acyl monoester as shown. The singlet at 1.98 ppm corresponds to the degradation product shown in the figure and confirmed by mass spec (see below). The presence of the undesired metabolite that has a ^{31}P NMR is a reflection of the low stability of these compounds. The experiment was carried out as reported previously by Osgerby et al. 2017, 60 (8), 3518–3524.

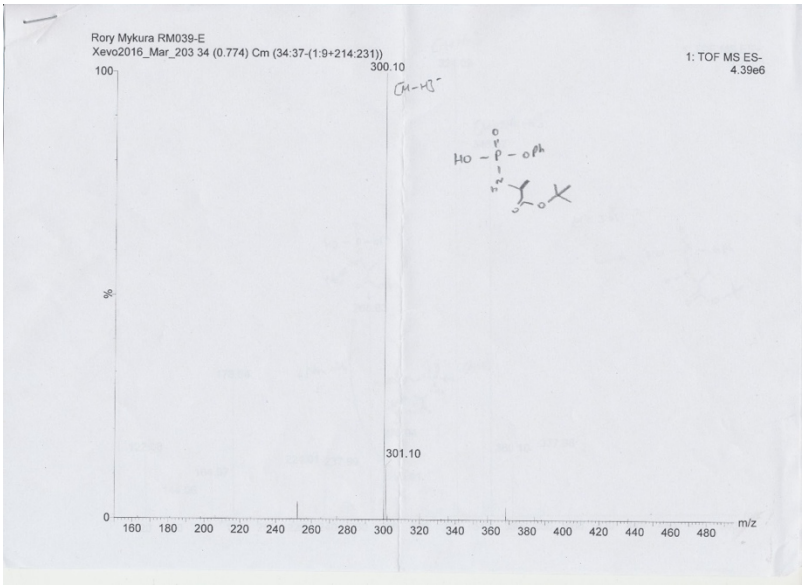
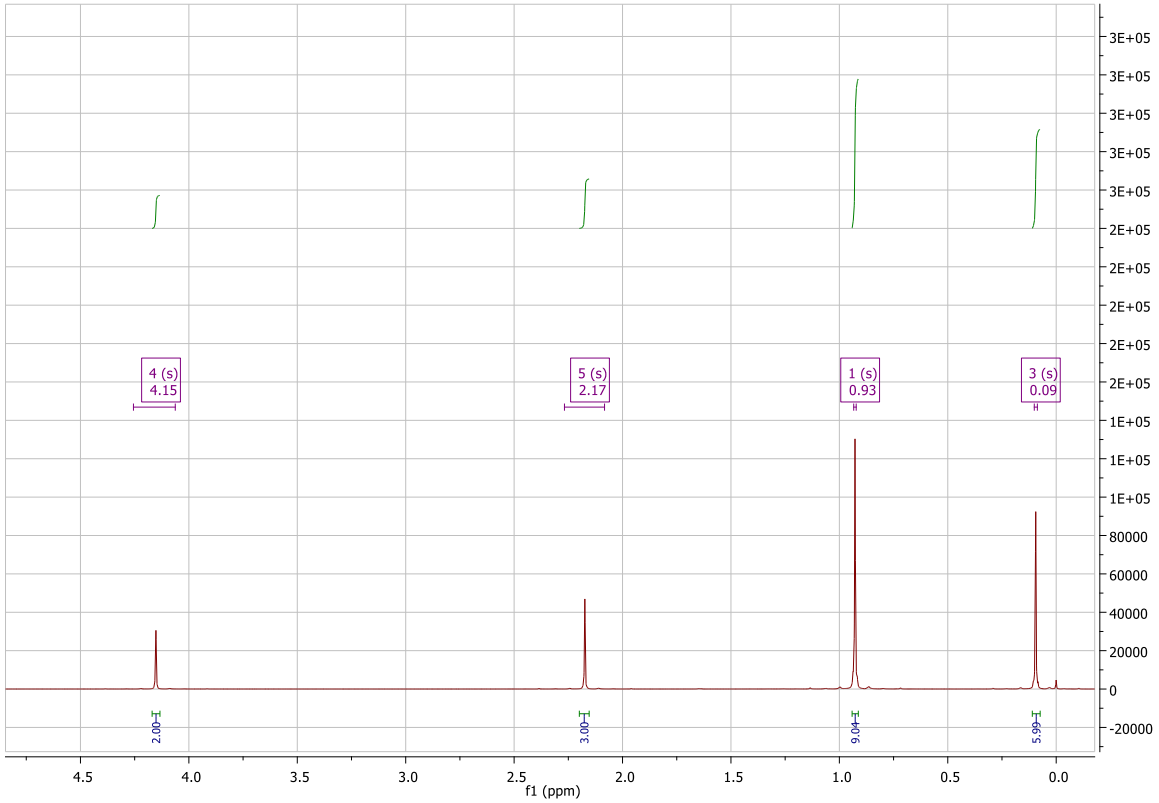


Figure S2. Negative ion spectrum for HMBP ProPAgen 6d following incubation with cathepsin A for 72 hours.

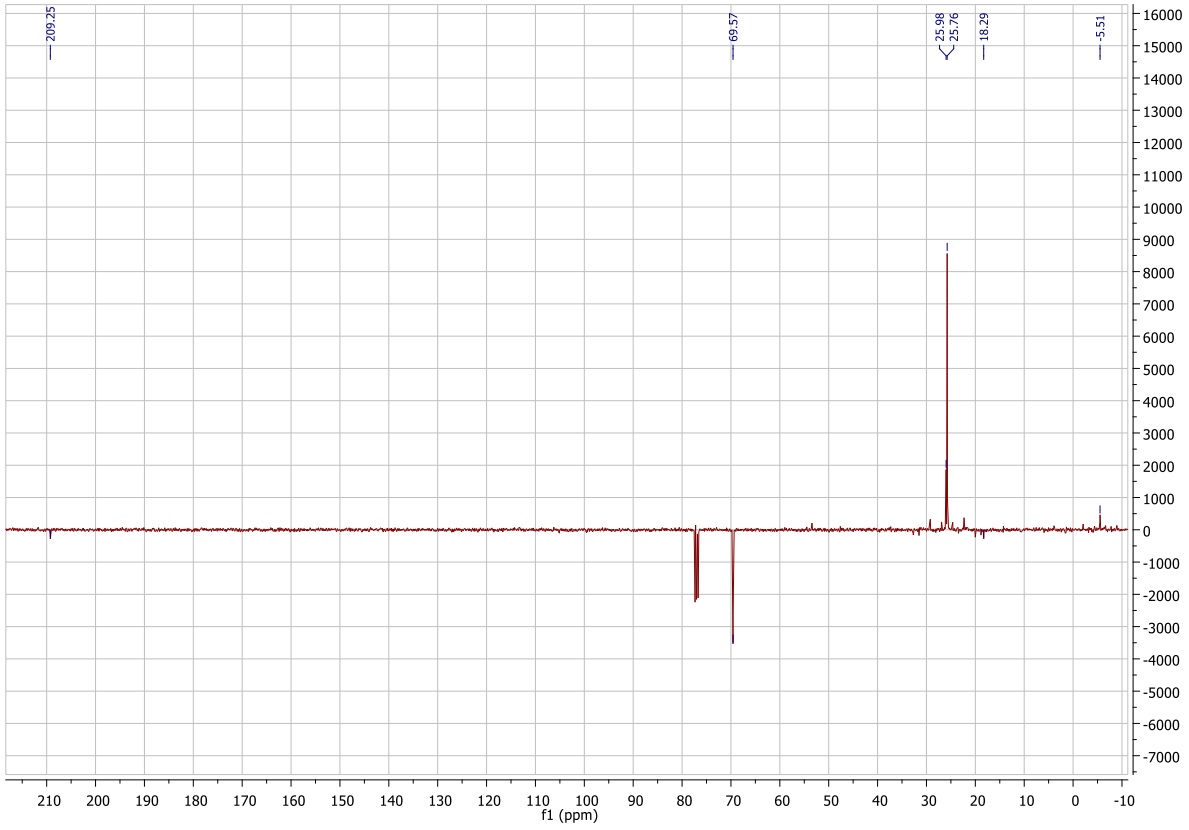
II. NMR spectra

1-((tert-butyldimethylsilyl)oxy)propan-2-one, 2:

^{1}H NMR

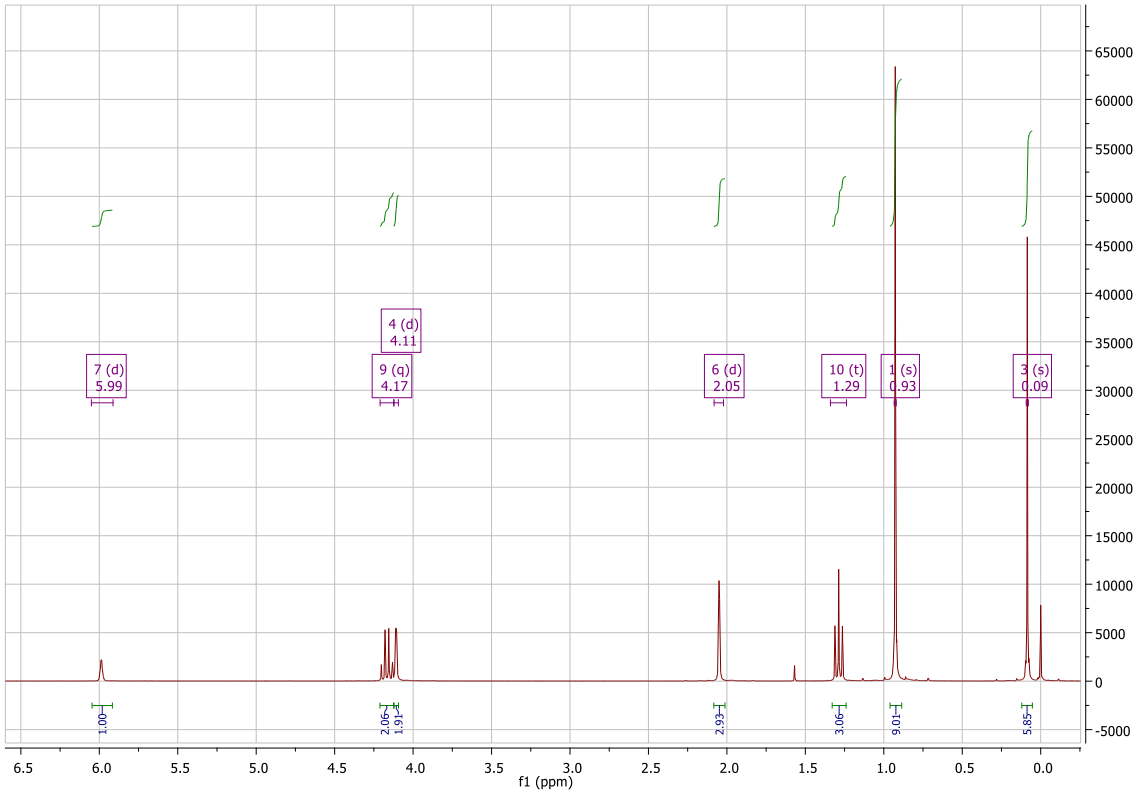


^{13}C NMR



Ethyl (*E*)-4-((tert-butyldimethylsilyl)oxy)-3-methylbut-2-enoate, 3:

^{1}H NMR



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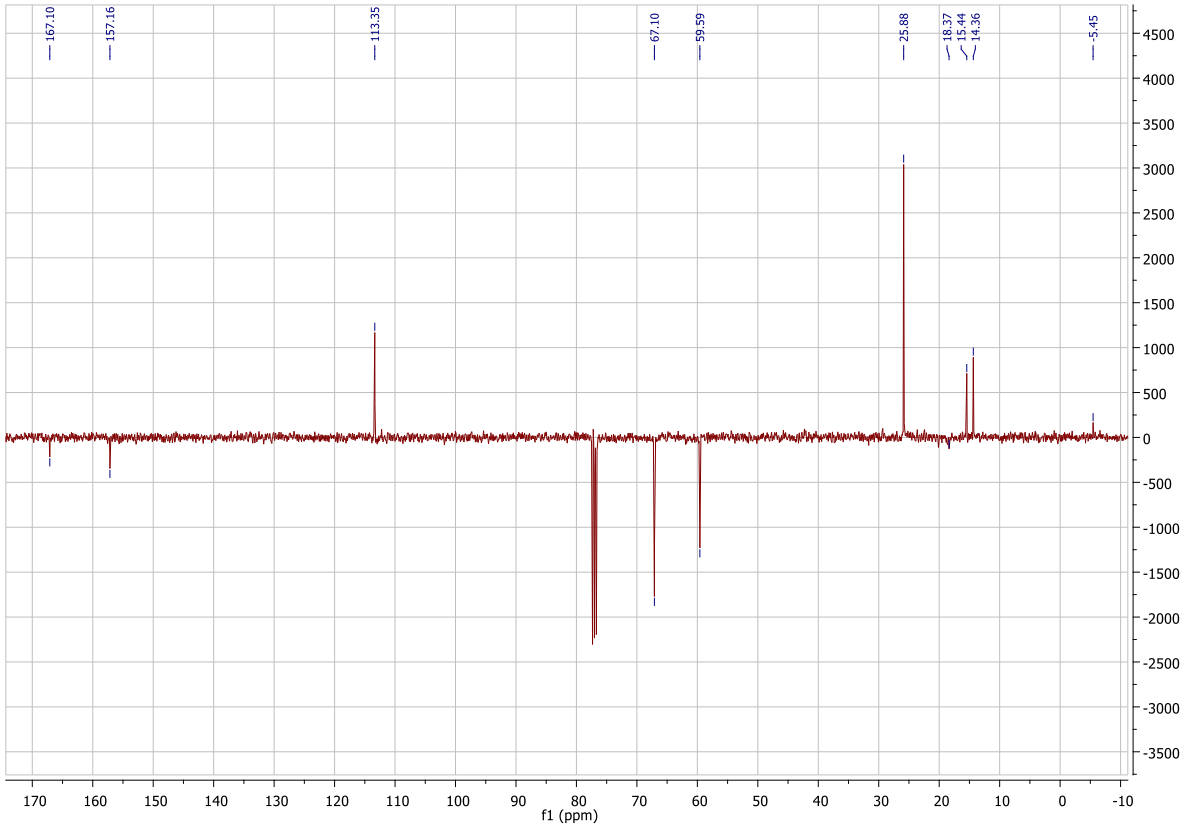
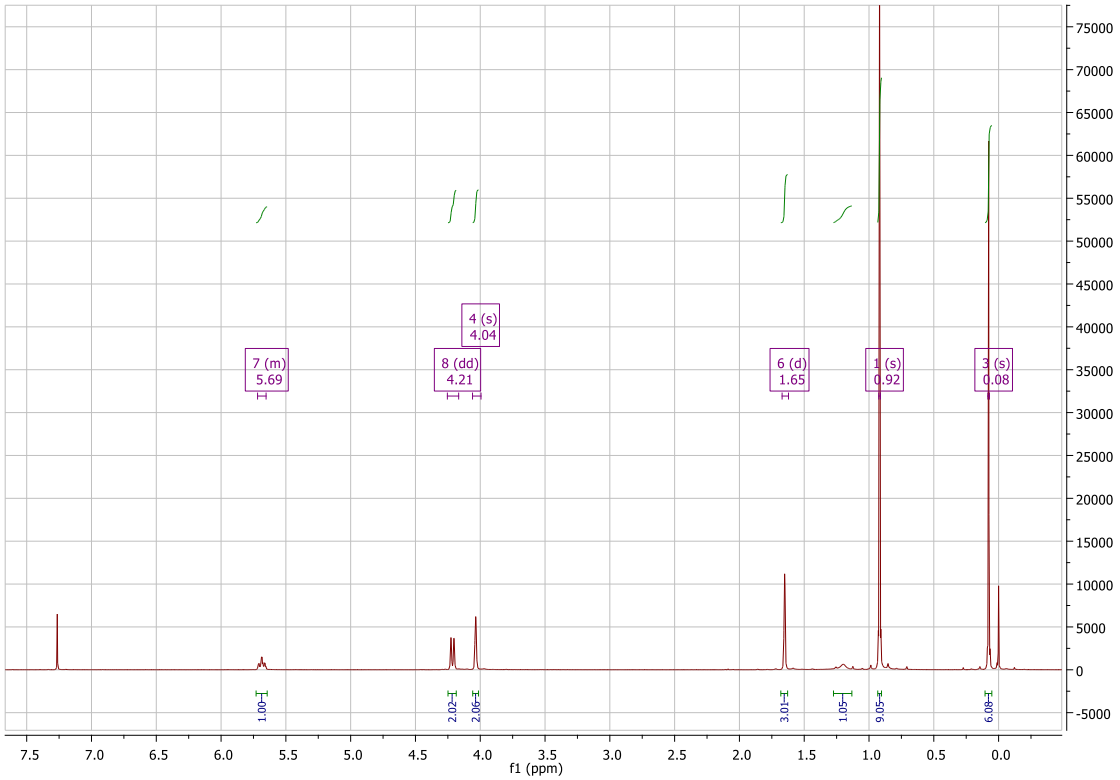
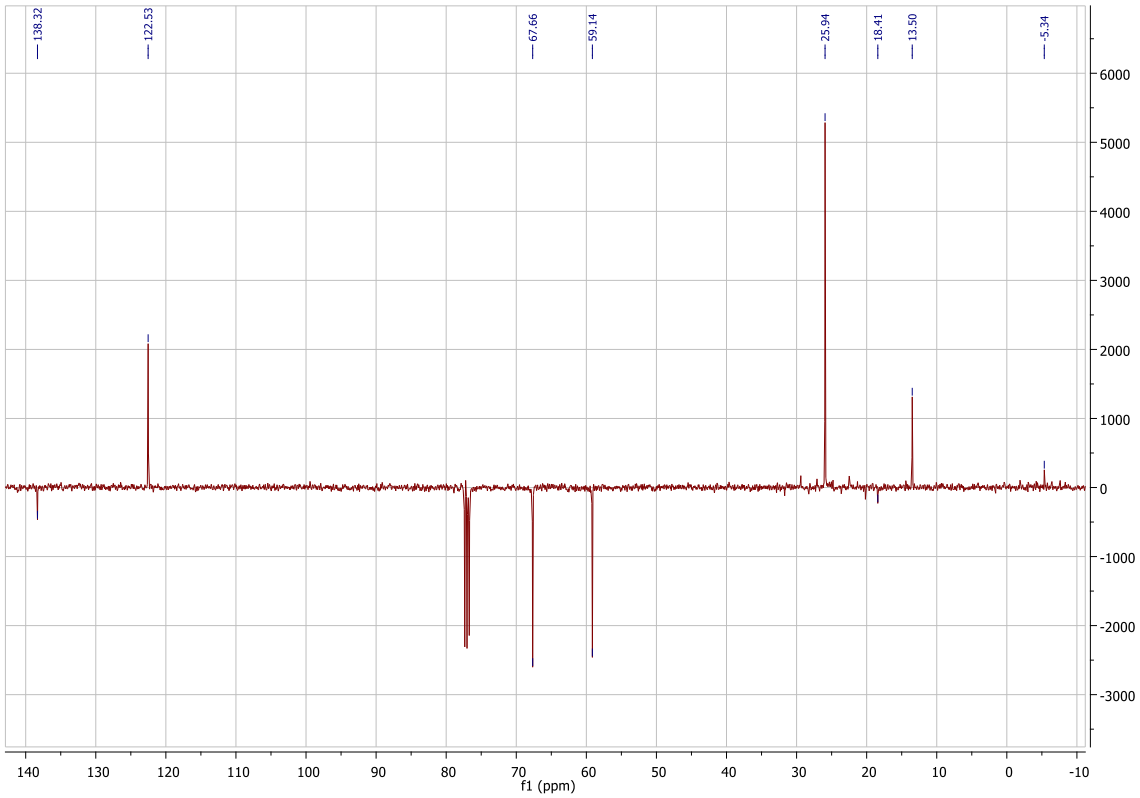


(*E*)-4-((tert-butyldimethylsilyl)oxy)-3-methylbut-2-en-1-ol, 4:

^{1}H NMR

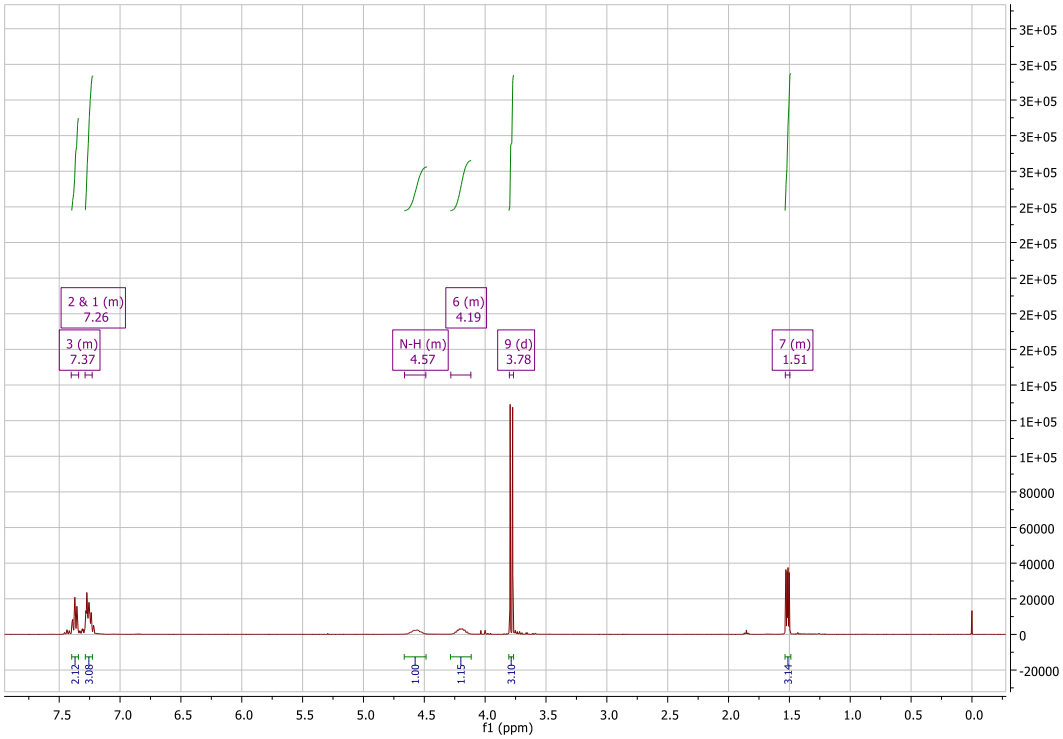


^{13}C NMR



Methyl (chloro(phenoxy)phosphoryl)-L-alaninate, 9a:

^{1}H NMR



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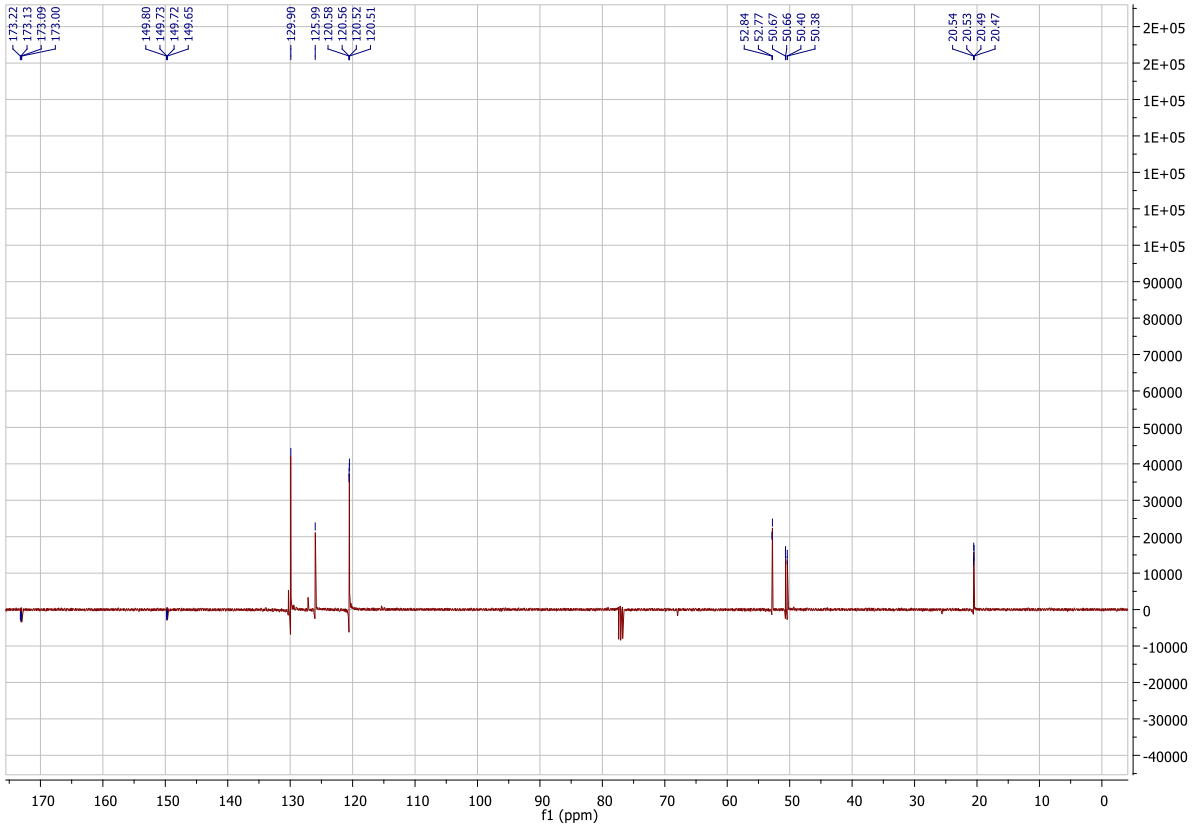
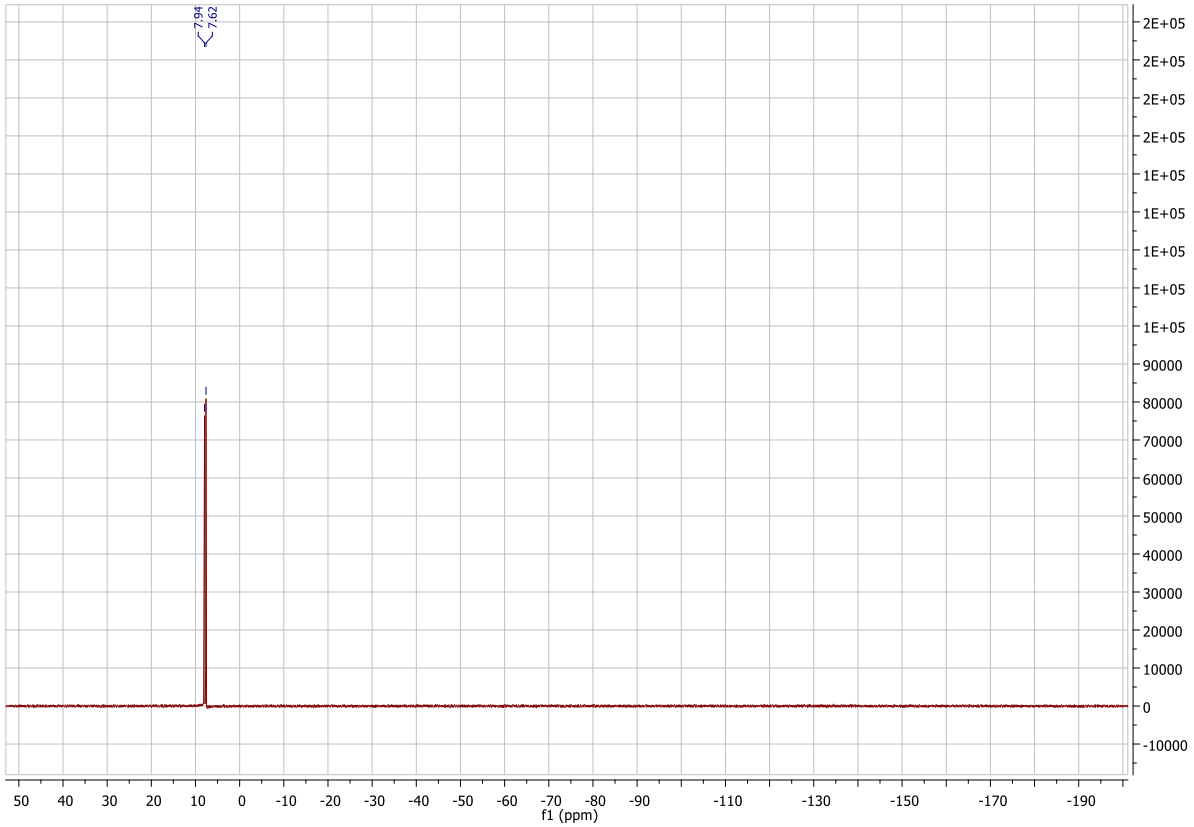
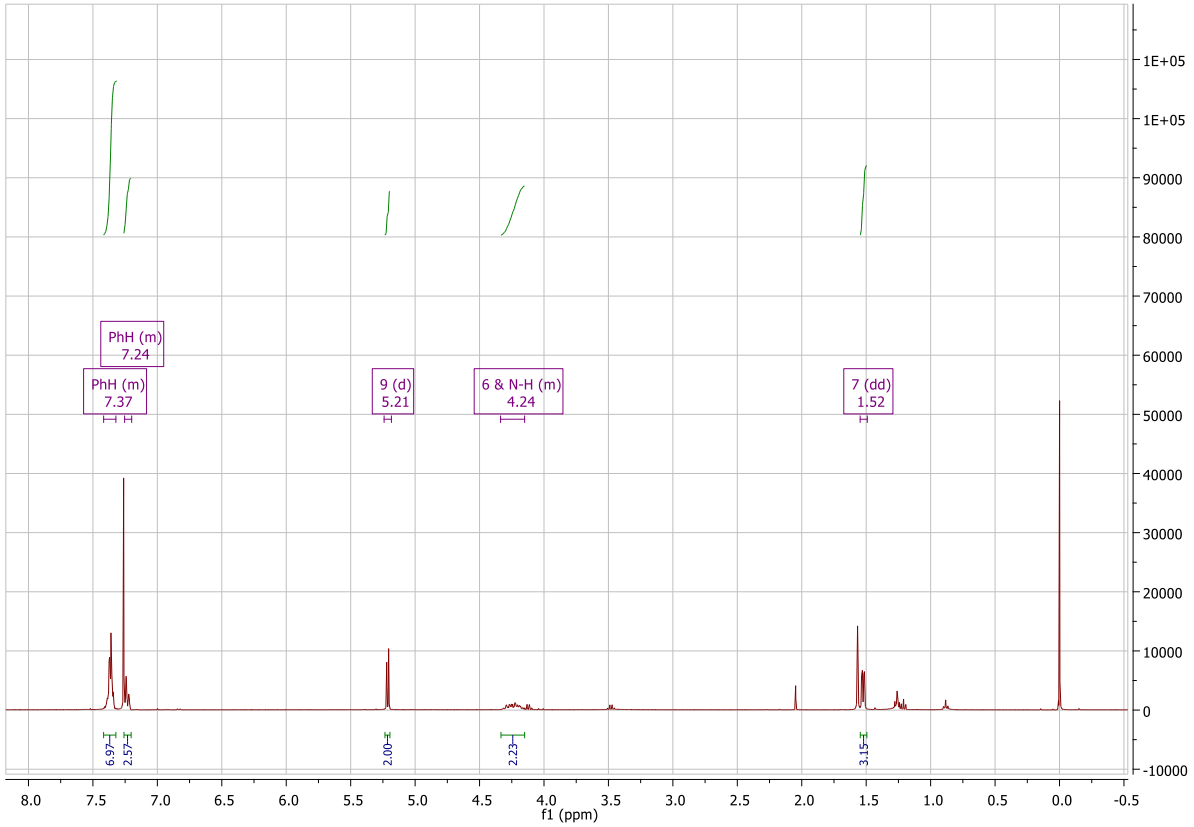


^{31}P NMR

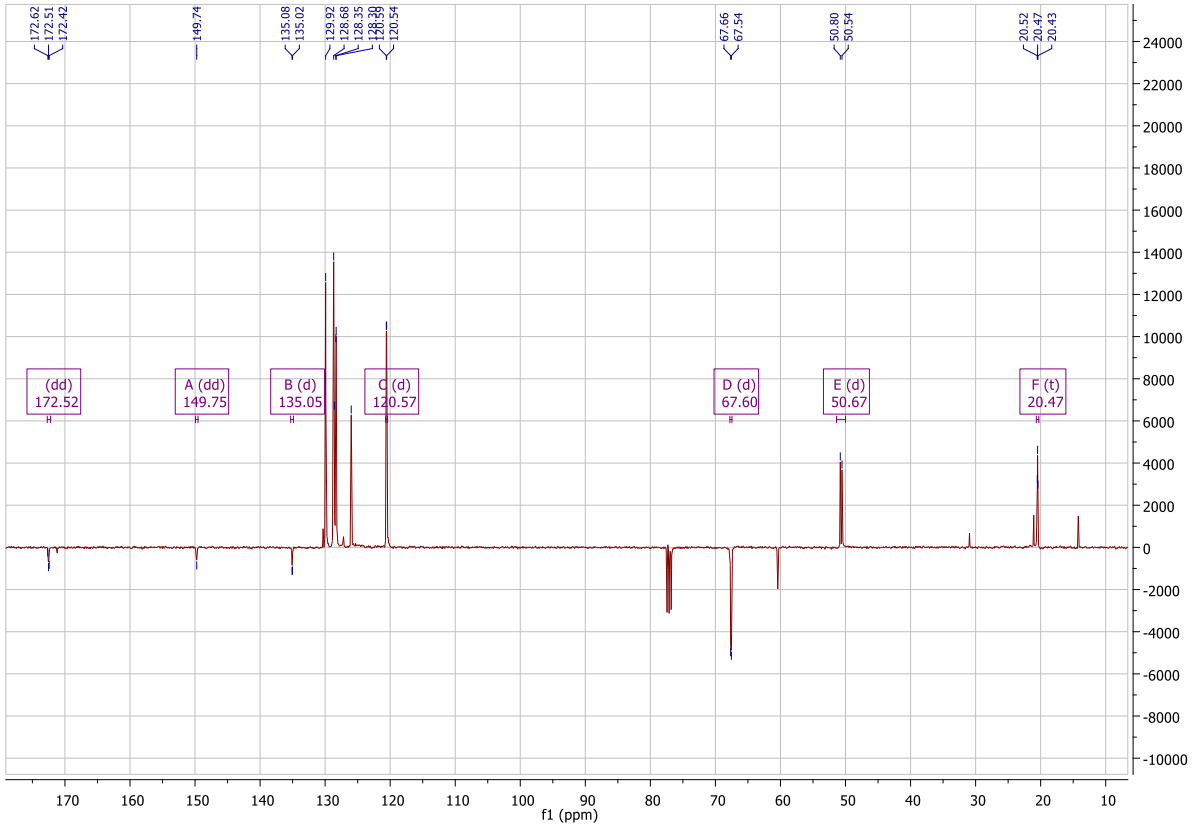


Benzyl (chloro(phenoxy)phosphoryl)-L-alaninate, 9b:

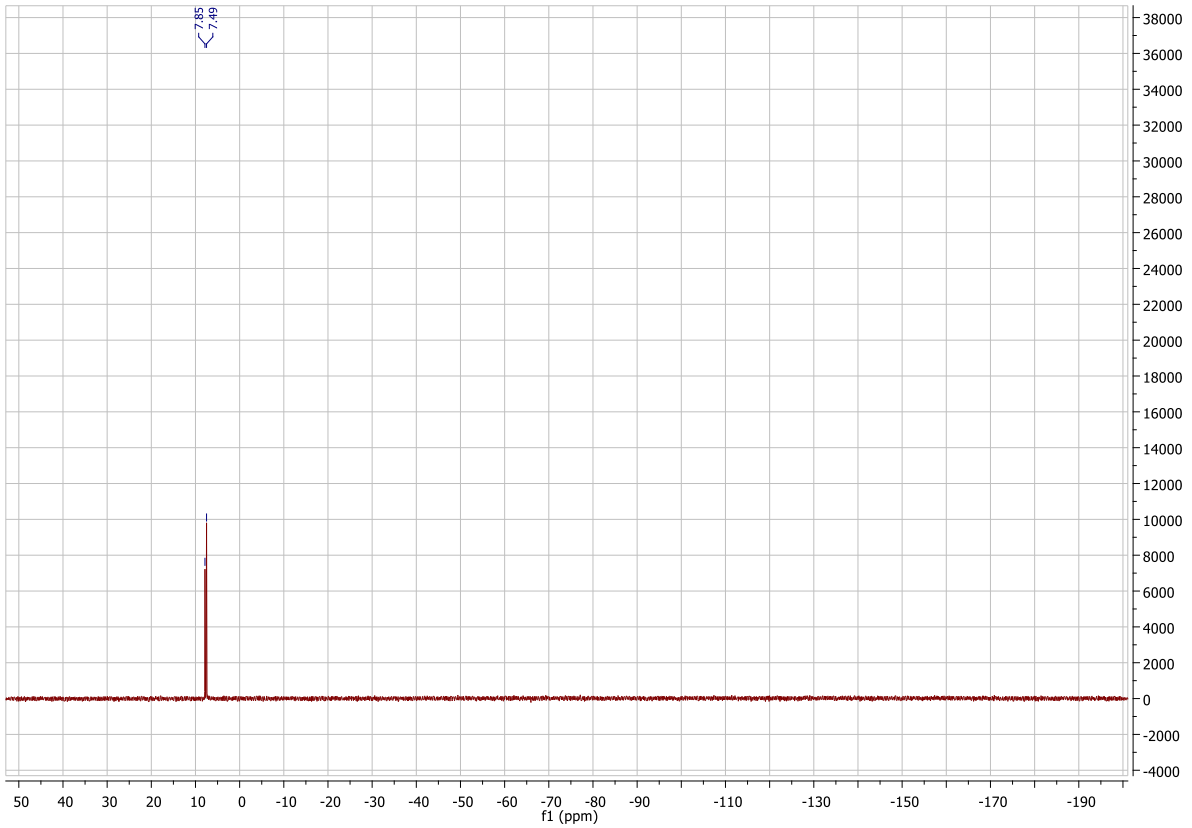
^{1}H NMR



^{13}C NMR

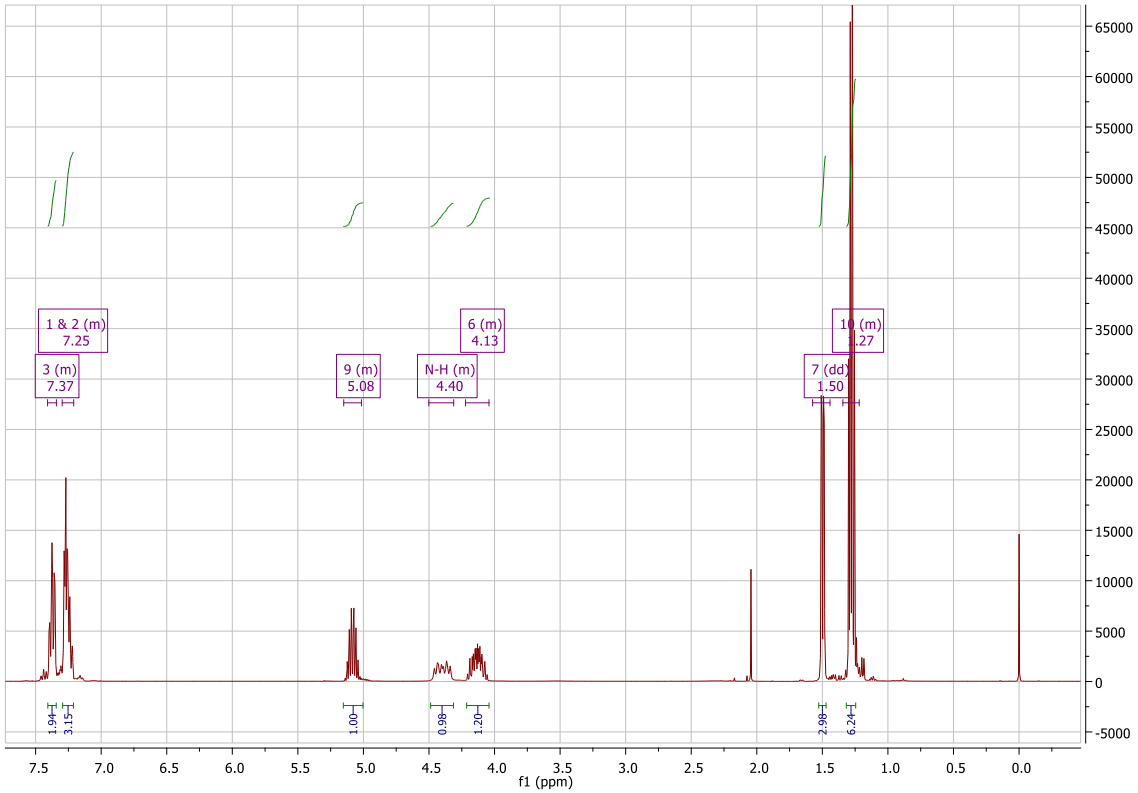


^{31}P NMR



Isopropyl (chloro(phenoxy)phosphoryl)-L-alaninate, 9c:

^{1}H NMR



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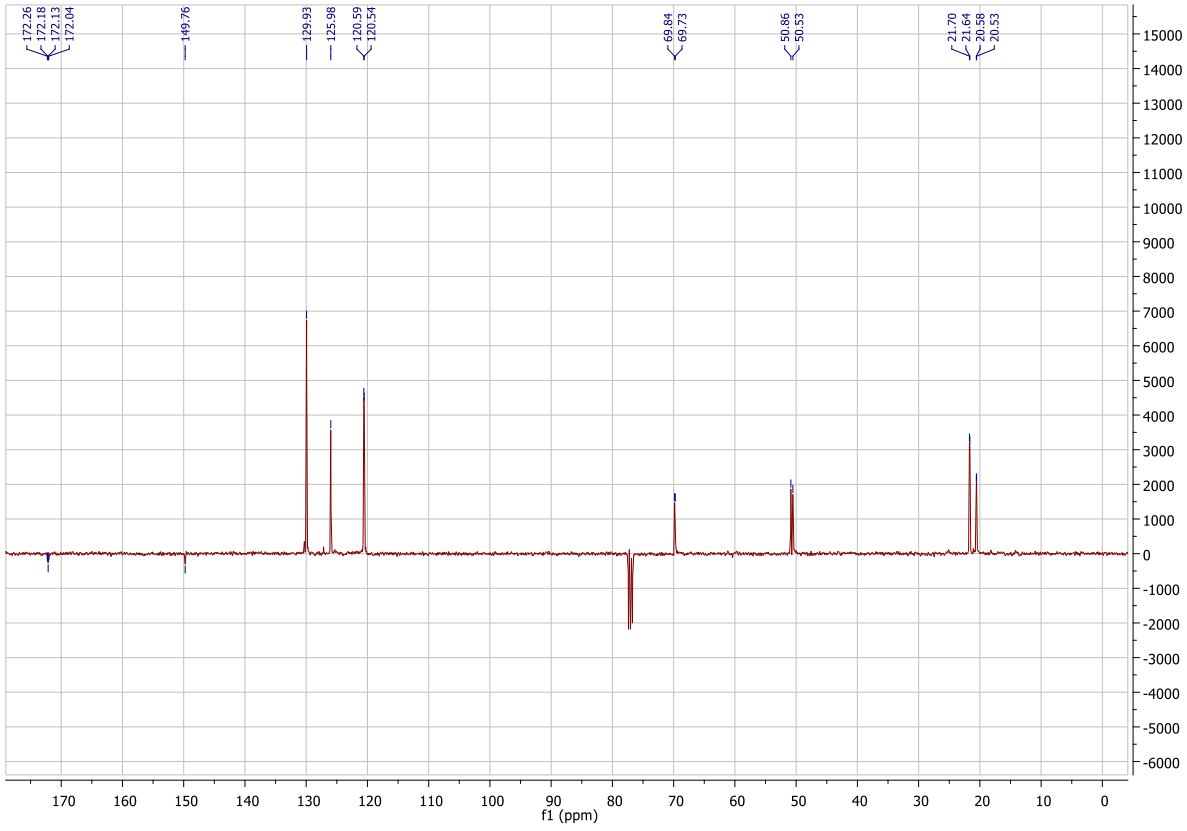

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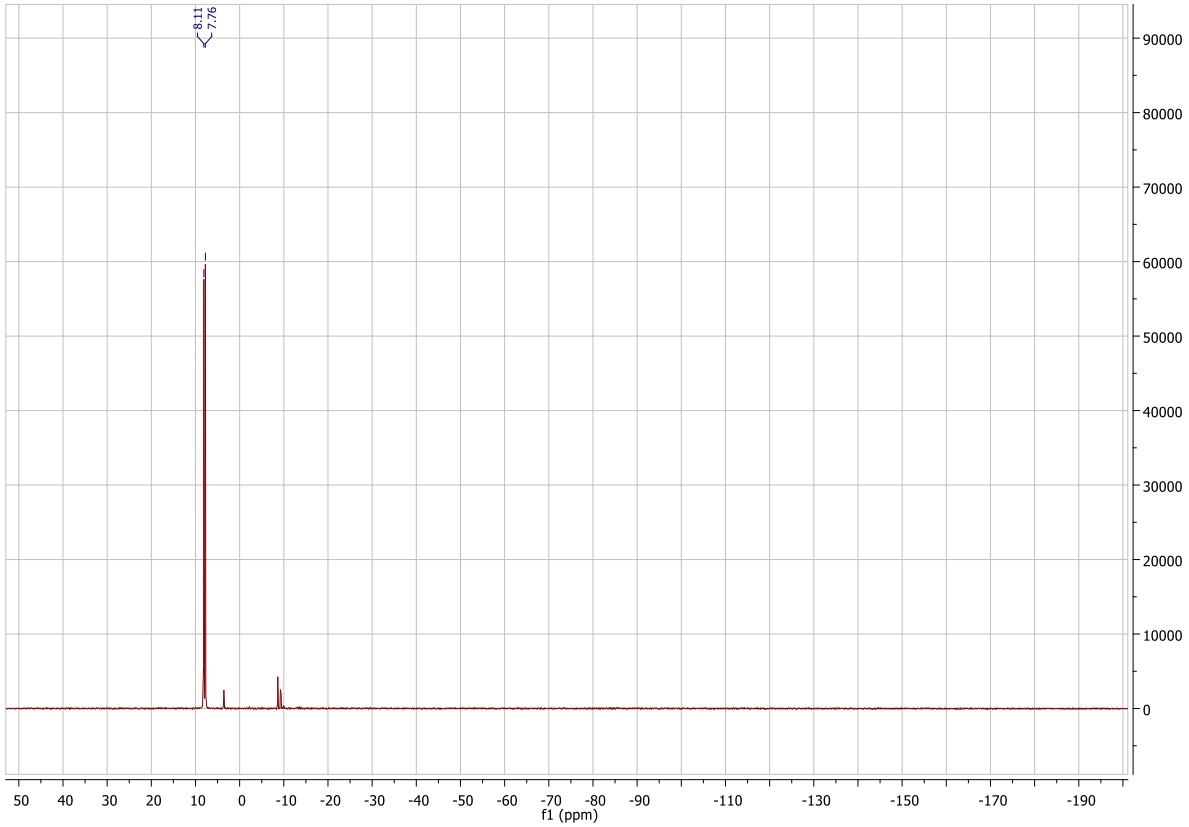

Tert-butyl (chloro(phenoxy)phosphoryl)-L-alaninate, 9d:

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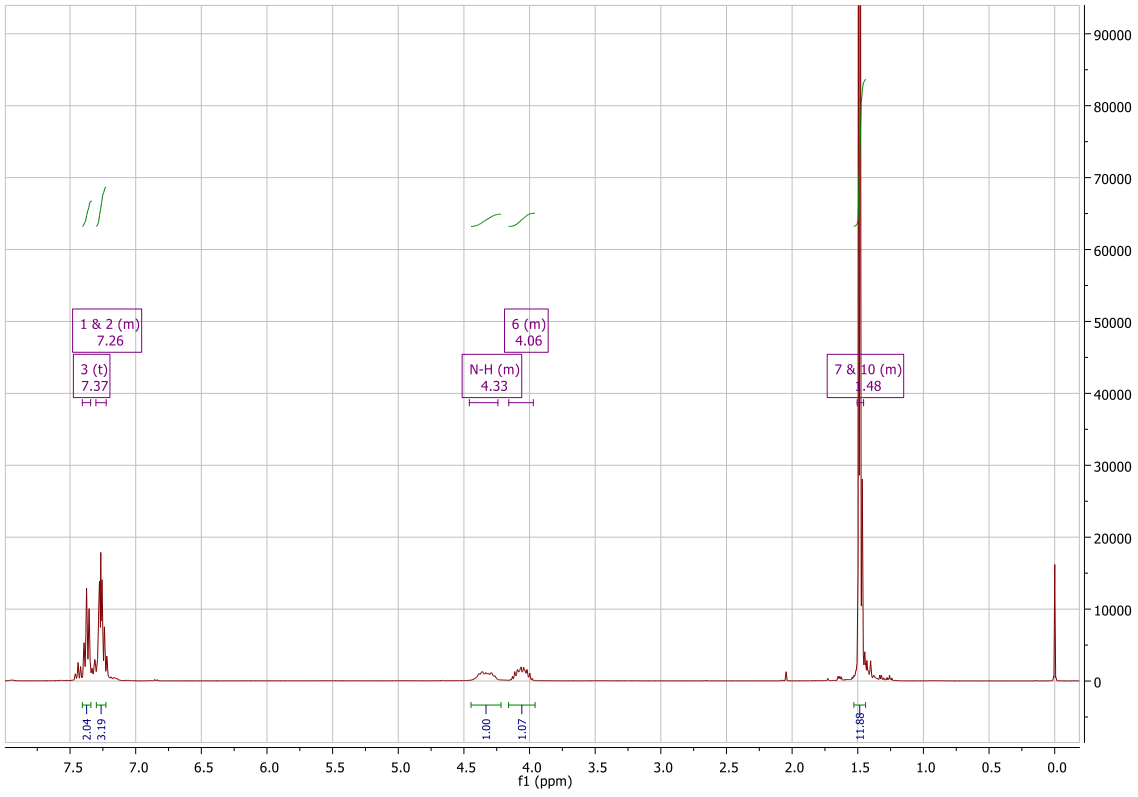

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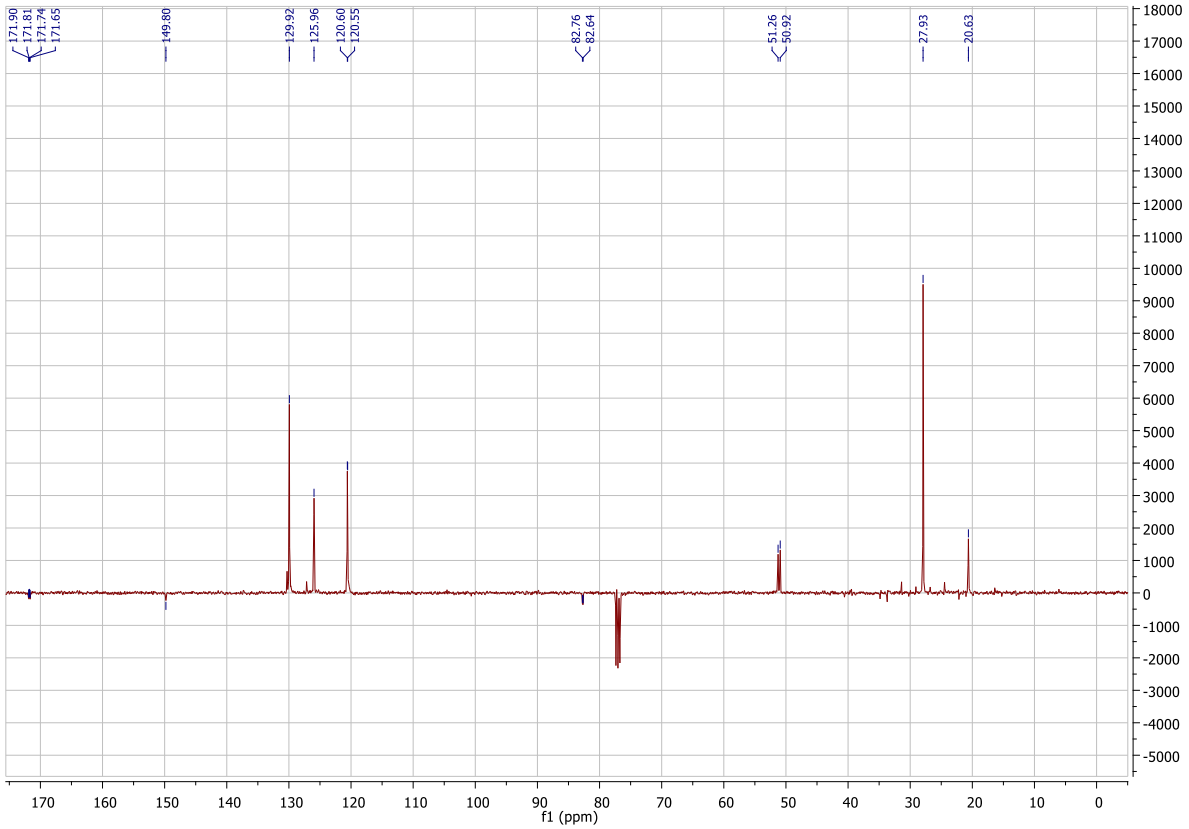

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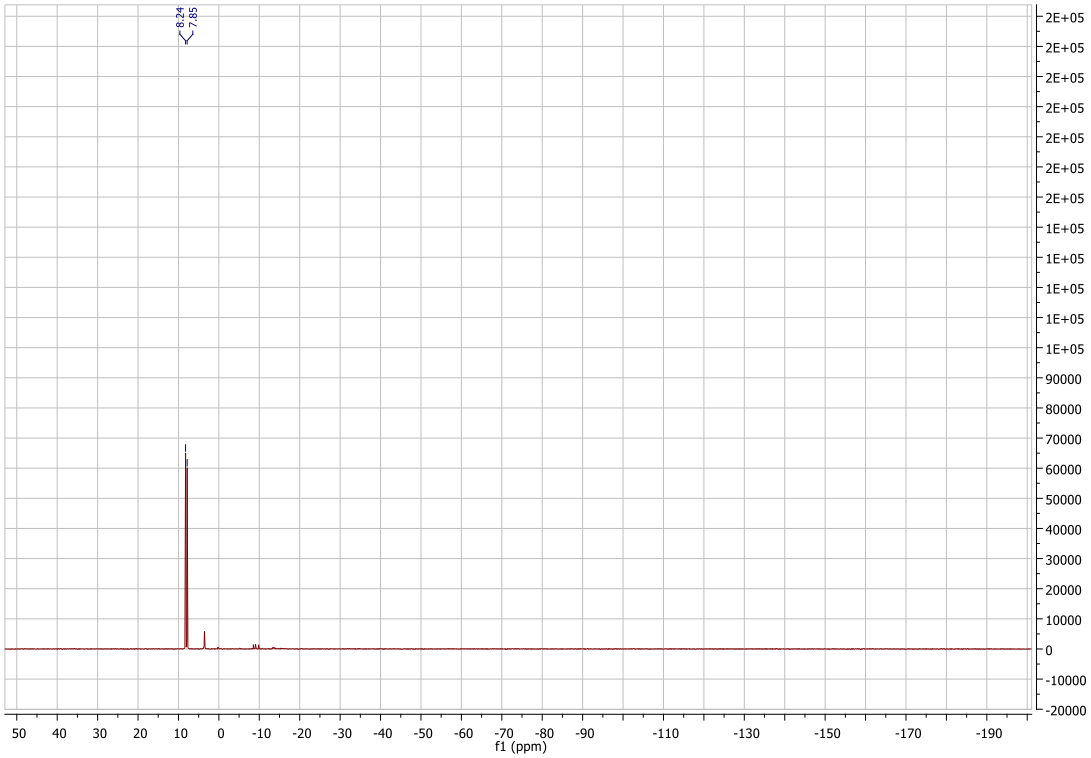
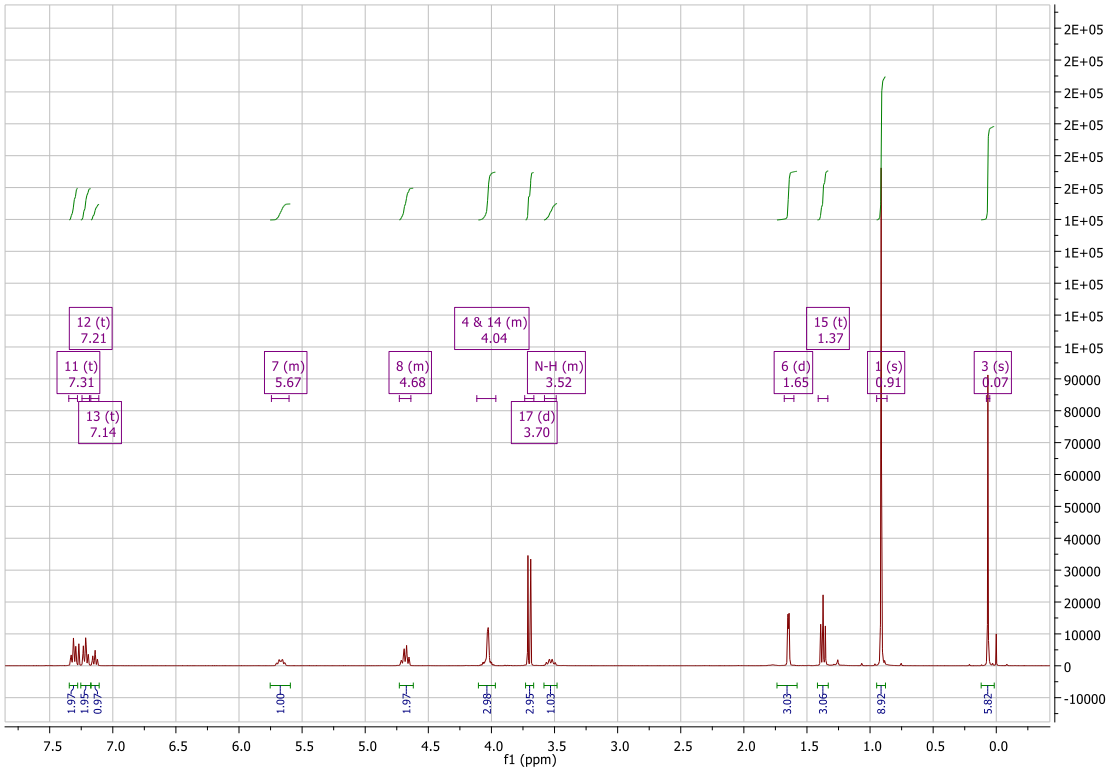


Methyl ((((E)-4-((tert-butyldimethylsilyl)oxy)-3-methylbut-2-en-1-yl)oxy)(phenoxy)phosphoryl)-L-alaninate, 5a:

^{1}H NMR



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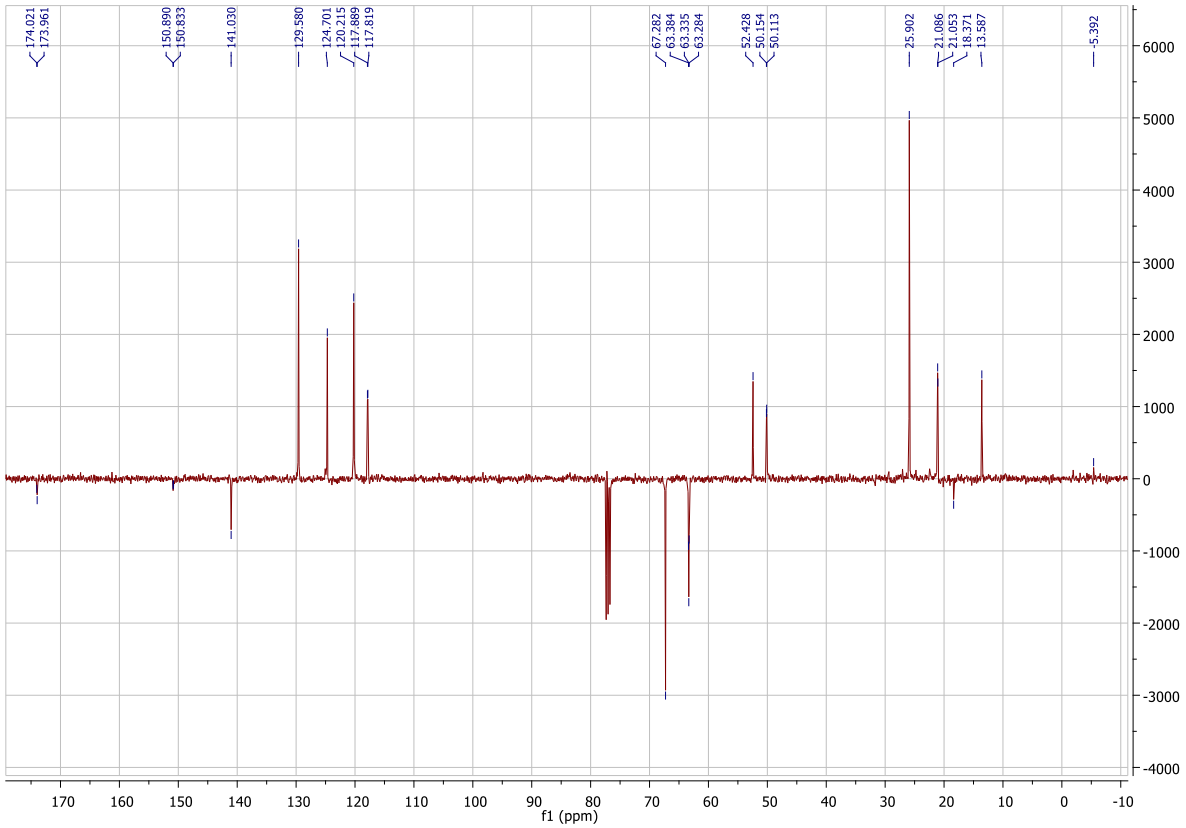

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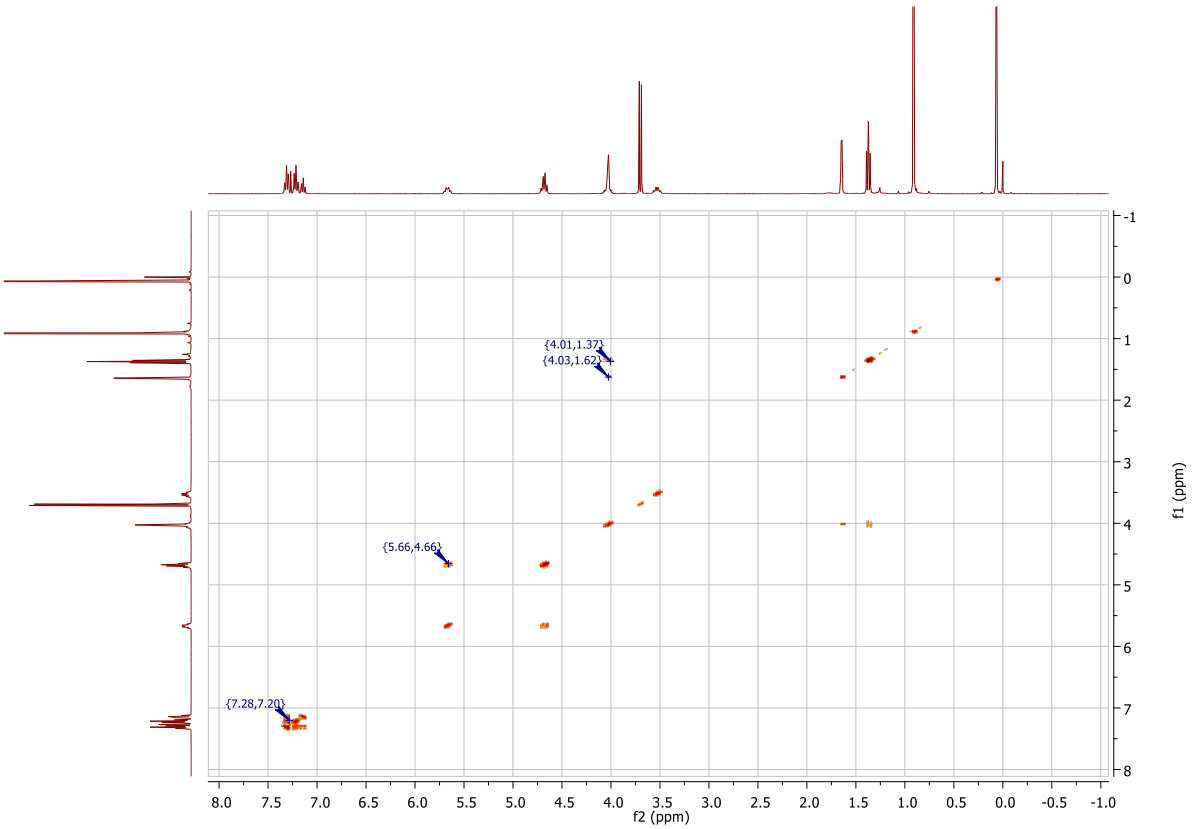

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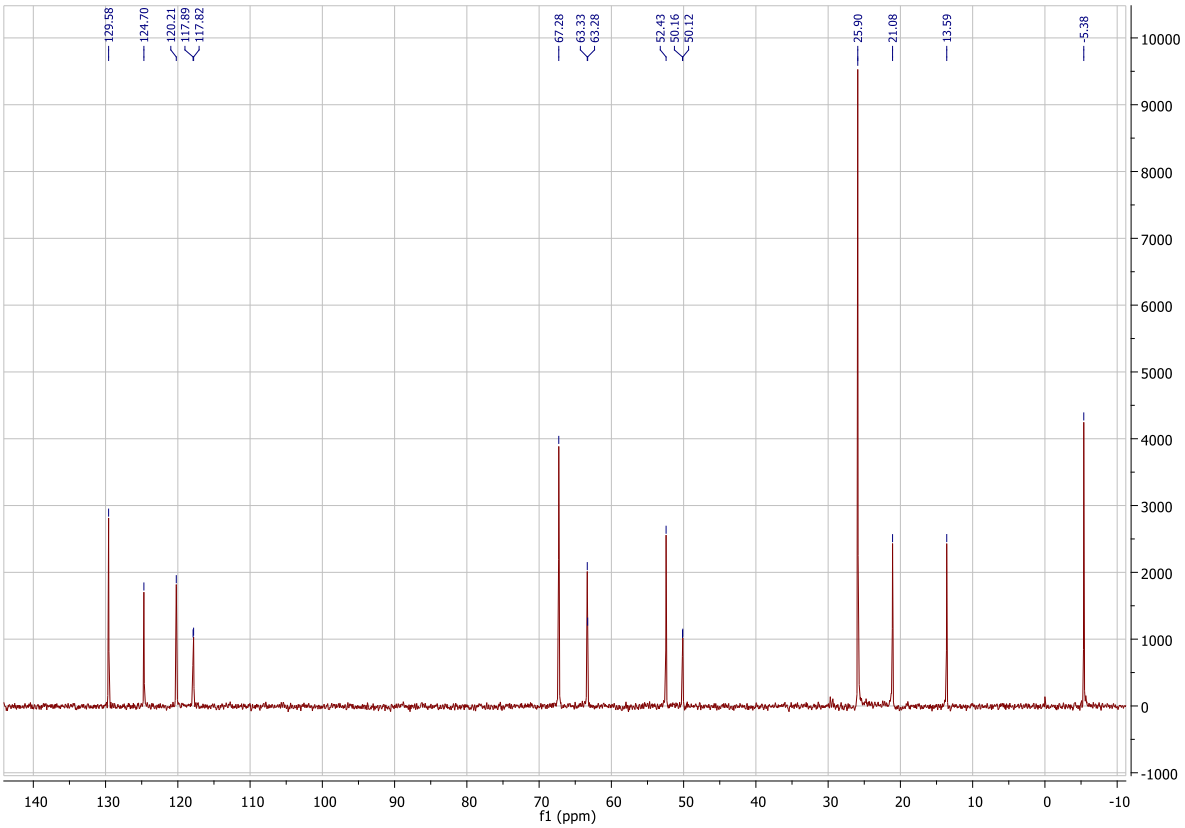

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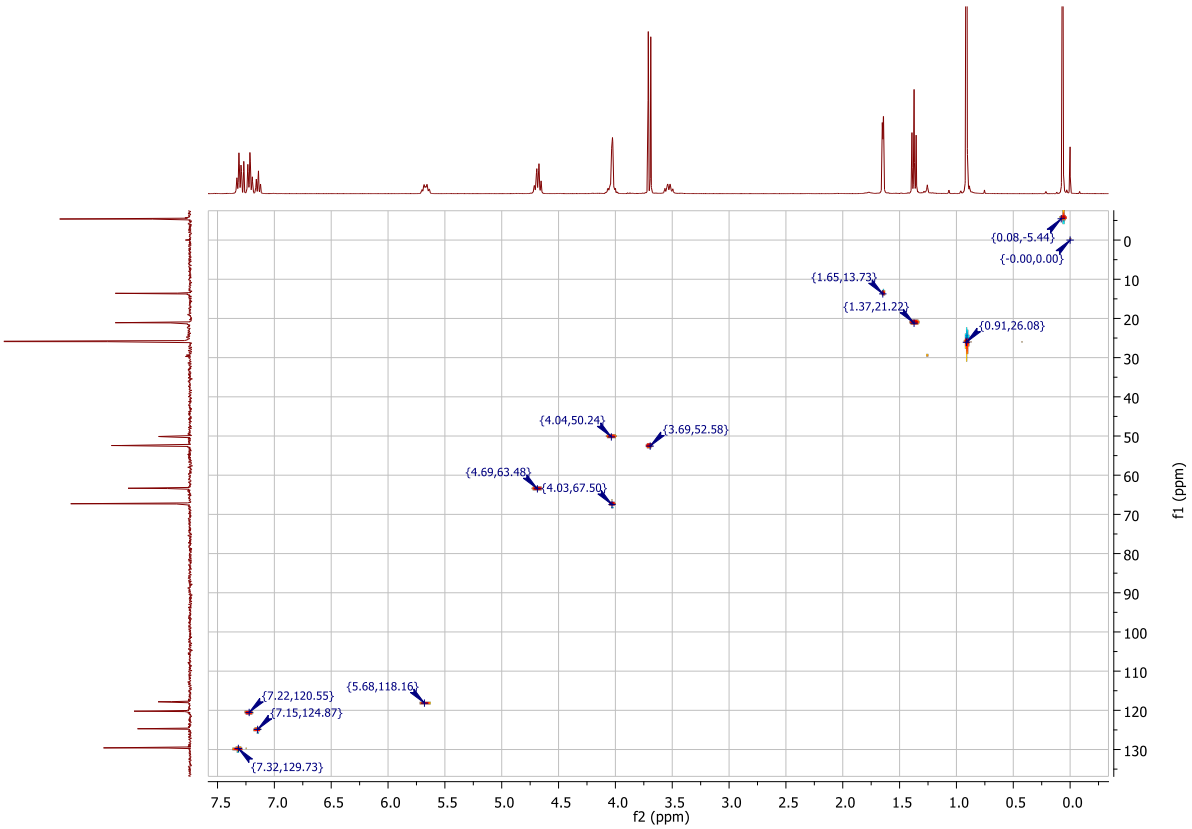

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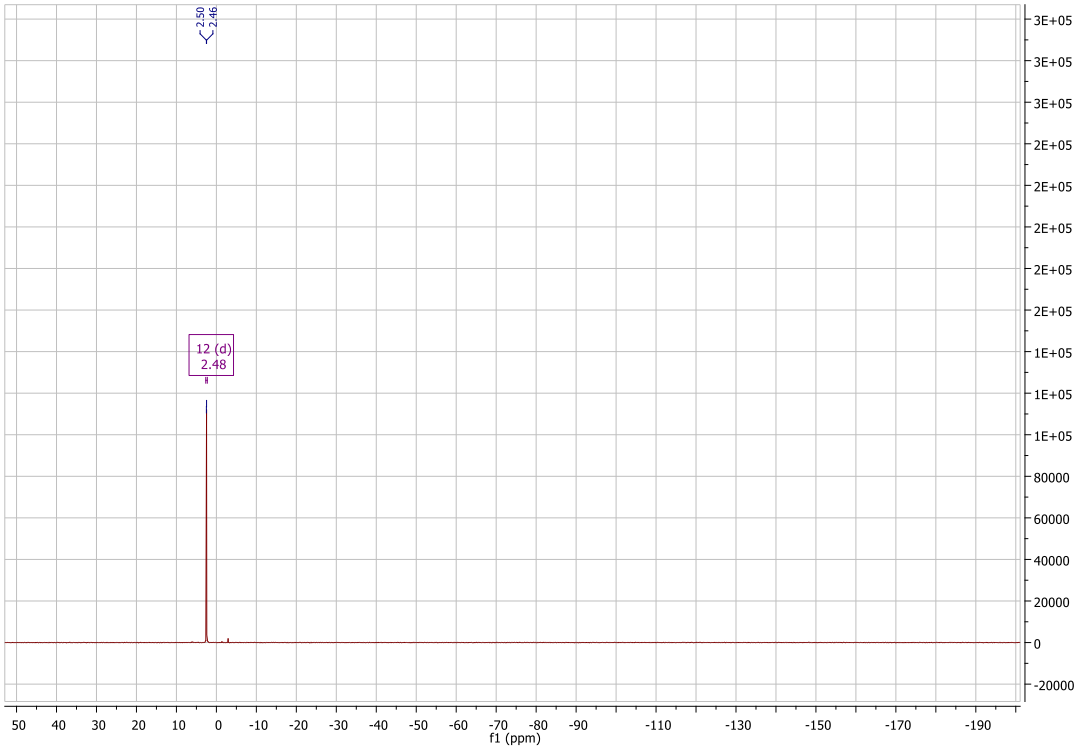

Benzyl ((((E)-4-((tert-butyldimethylsilyl)oxy)-3-methylbut-2-en-1-yl)oxy)(phenoxy)phosphoryl)-L-alaninate, 5b:

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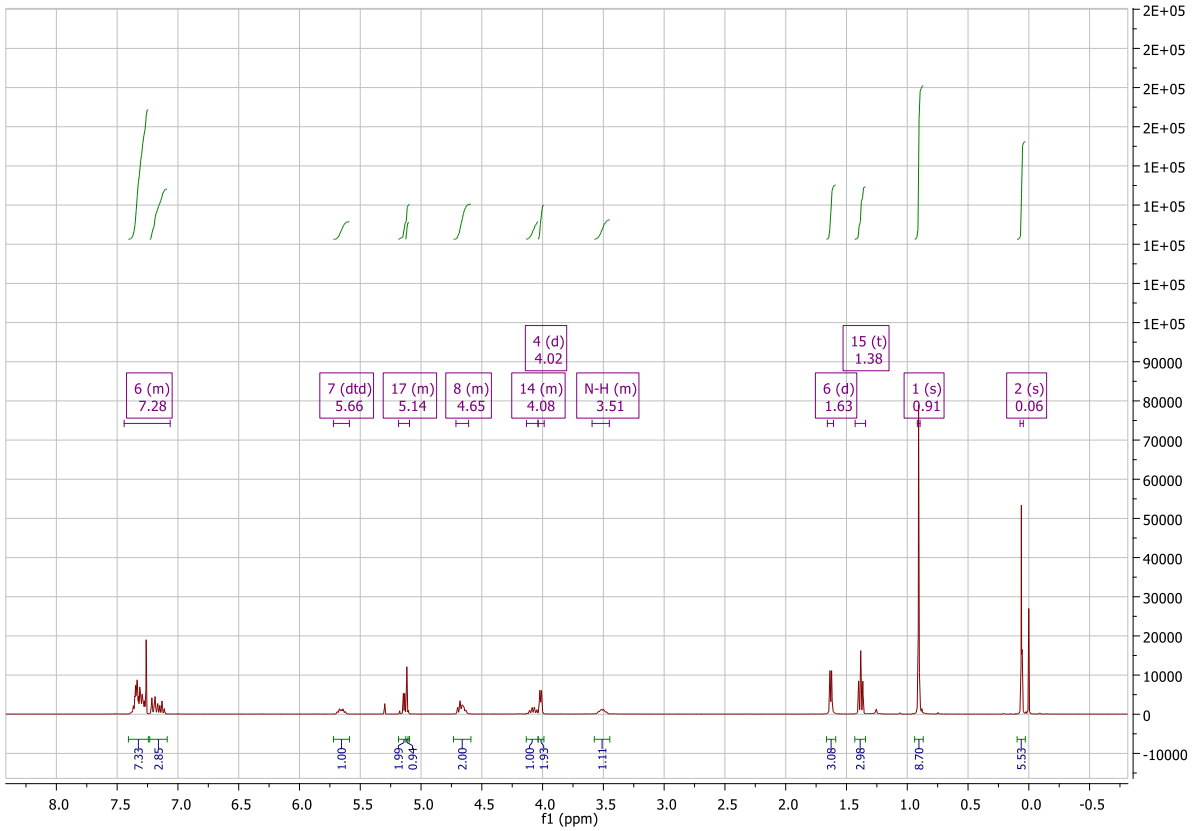

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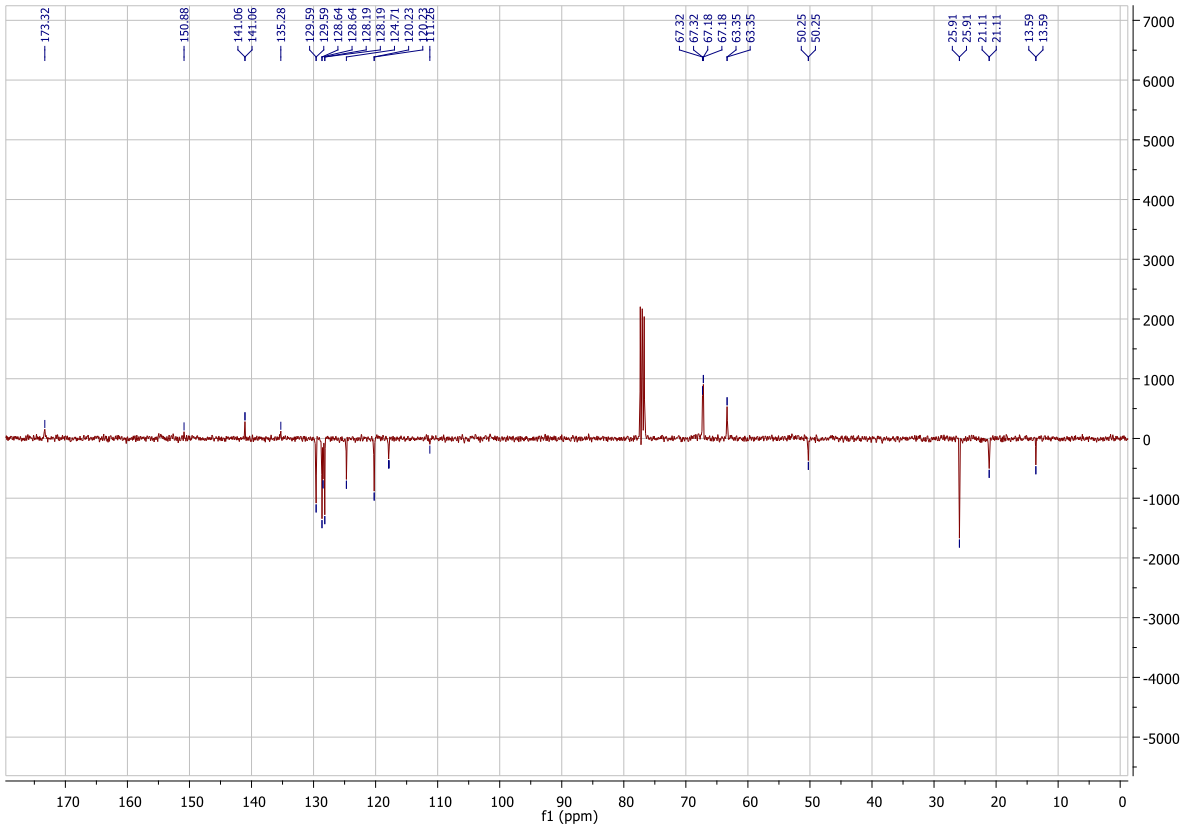

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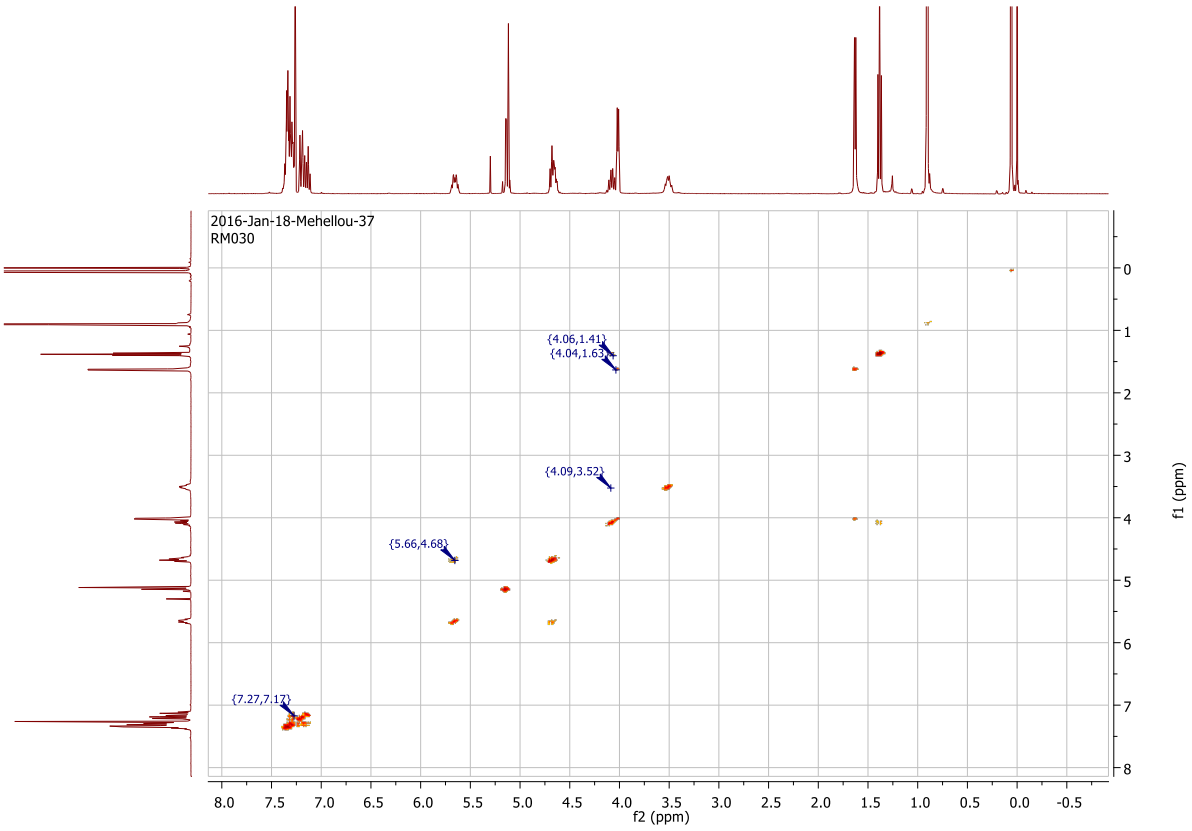

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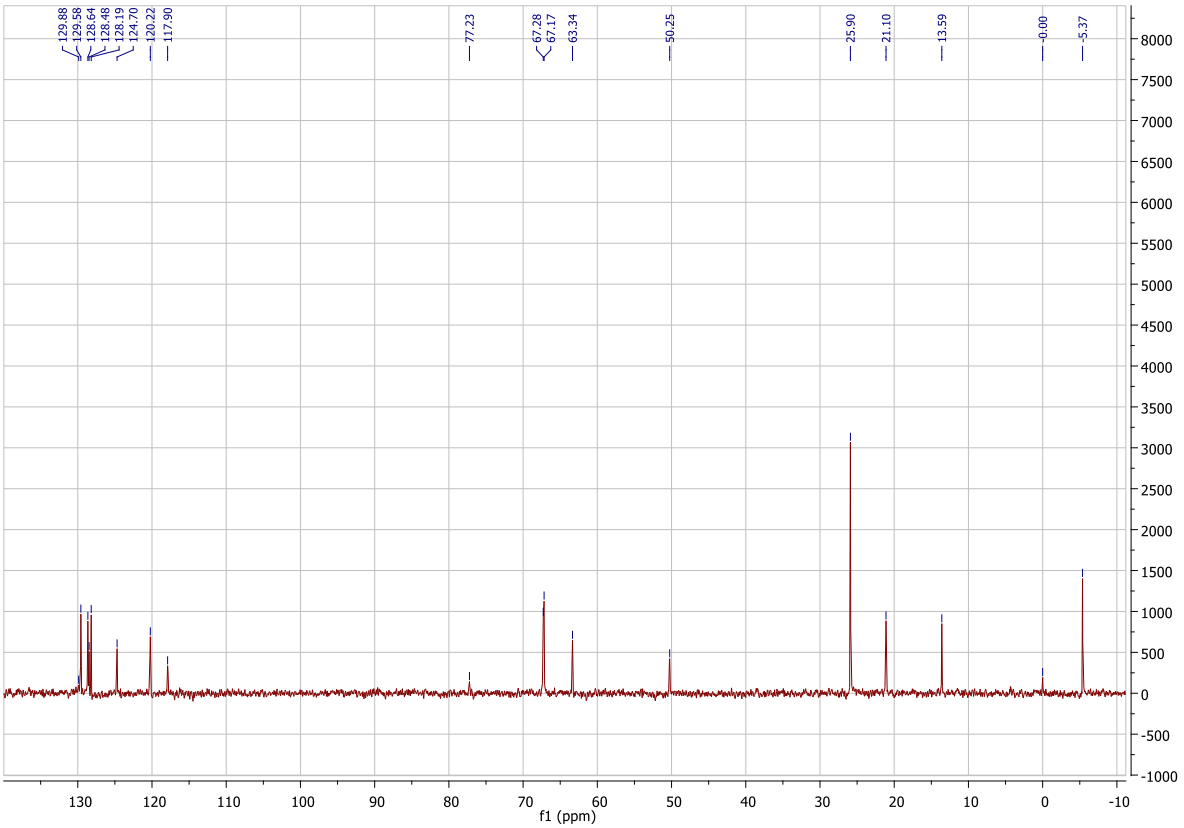

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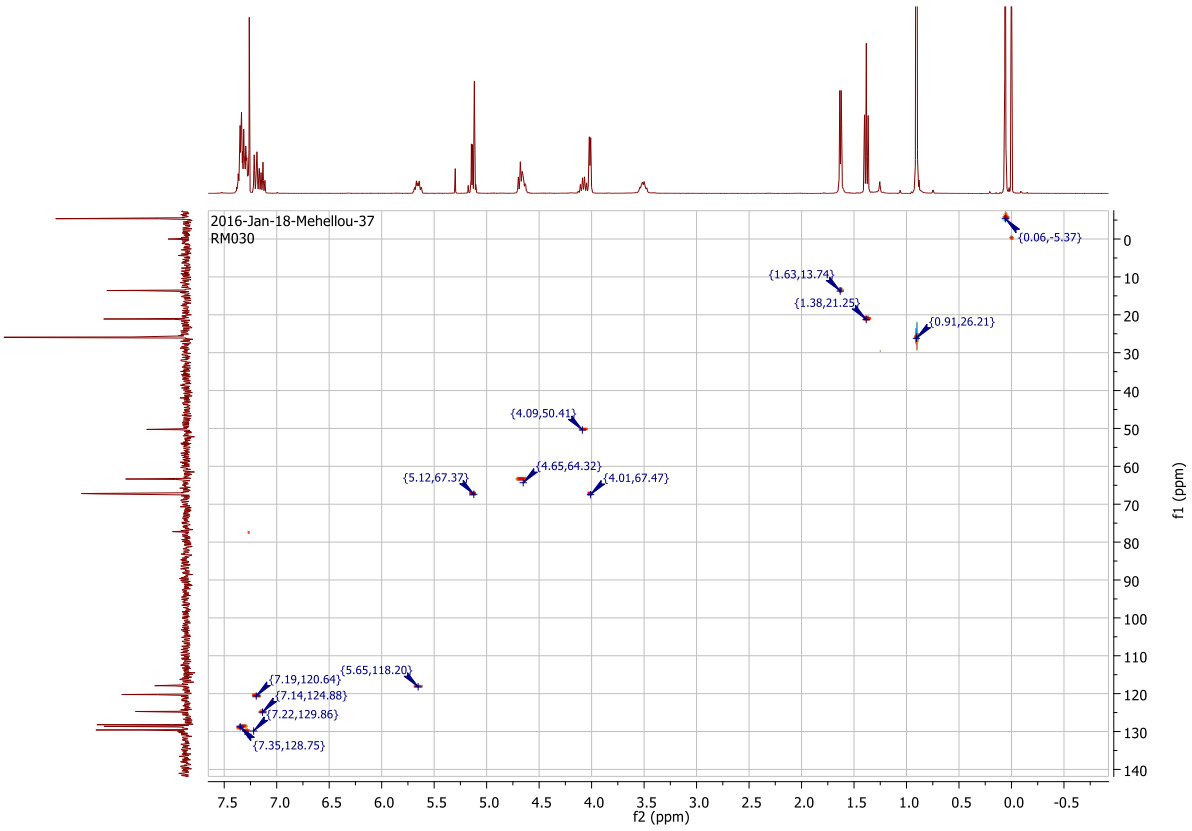
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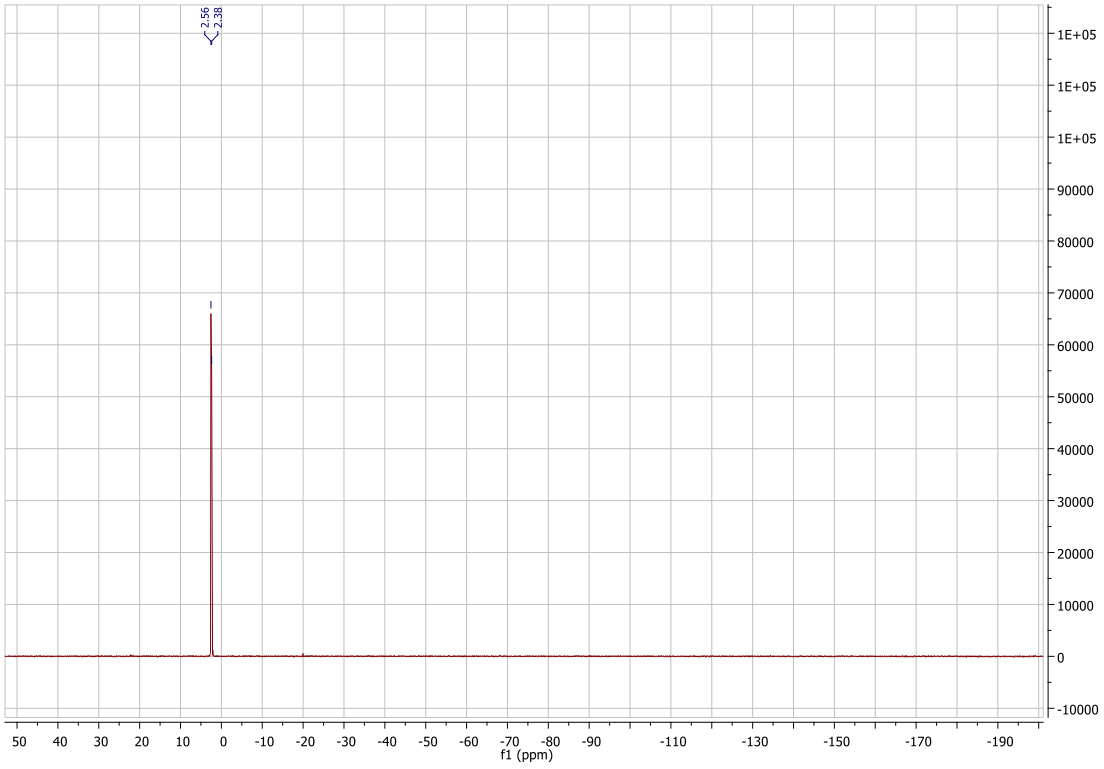
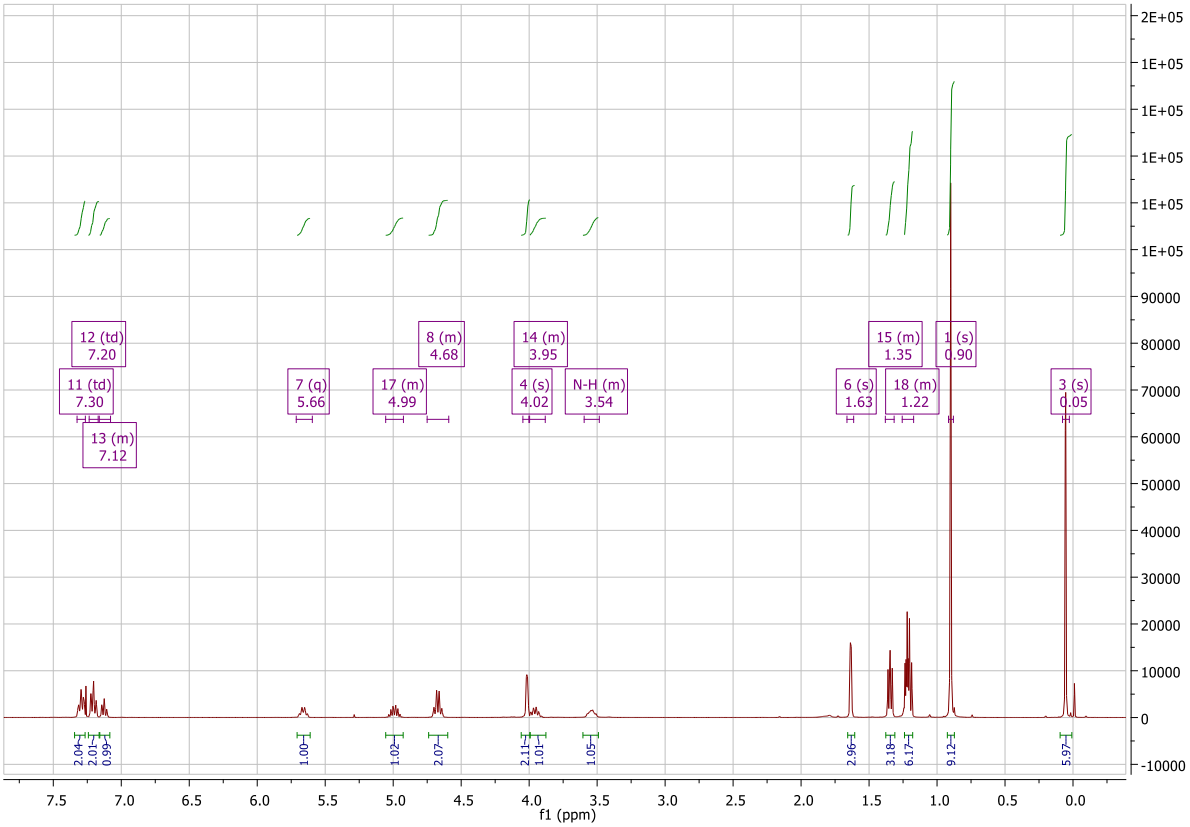
f2 (ppm)
f1 (ppm)

^{31}P NMR

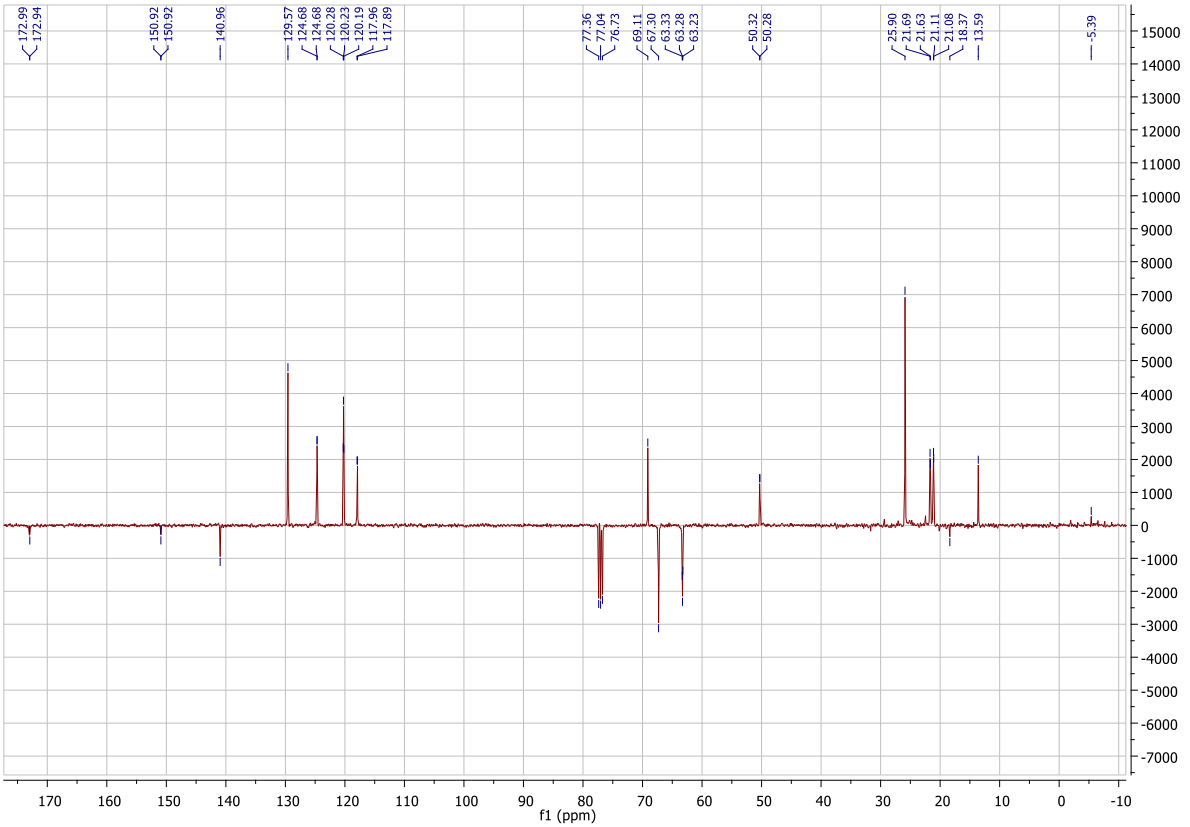

2.56
2.38
f1 (ppm)

Isopropyl ((((E)-4-((tert-butyldimethylsilyl)oxy)-3-methylbut-2-en-1-yl)oxy)(phenoxy)phosphoryl)-L-alaninate, 5c:

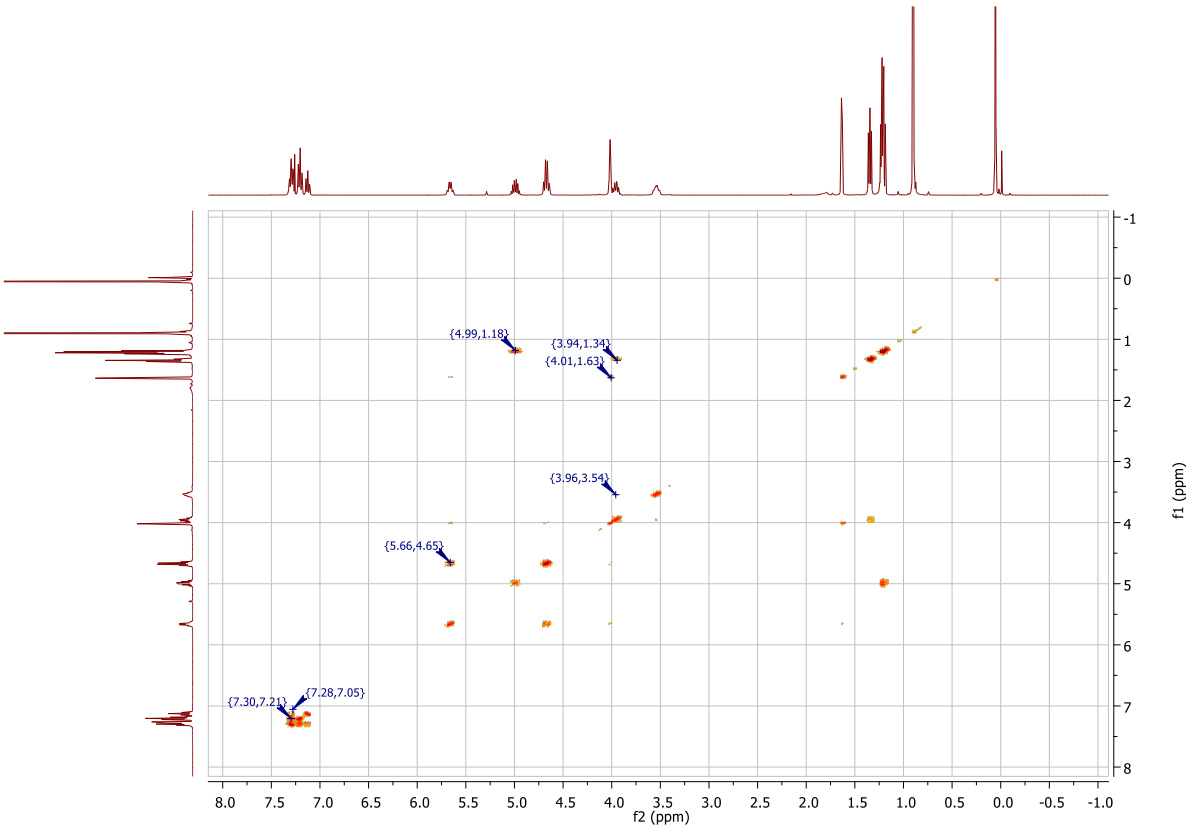
^{1}H NMR



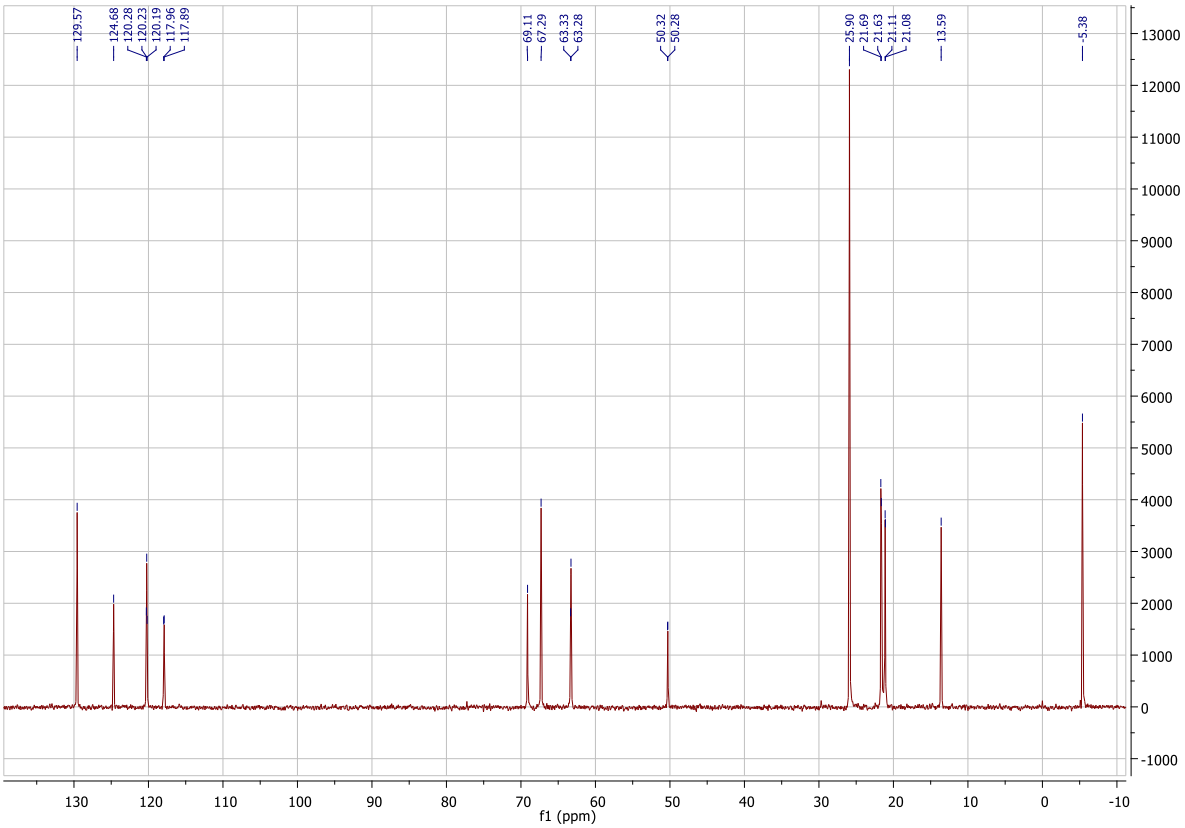
^{13}C NMR



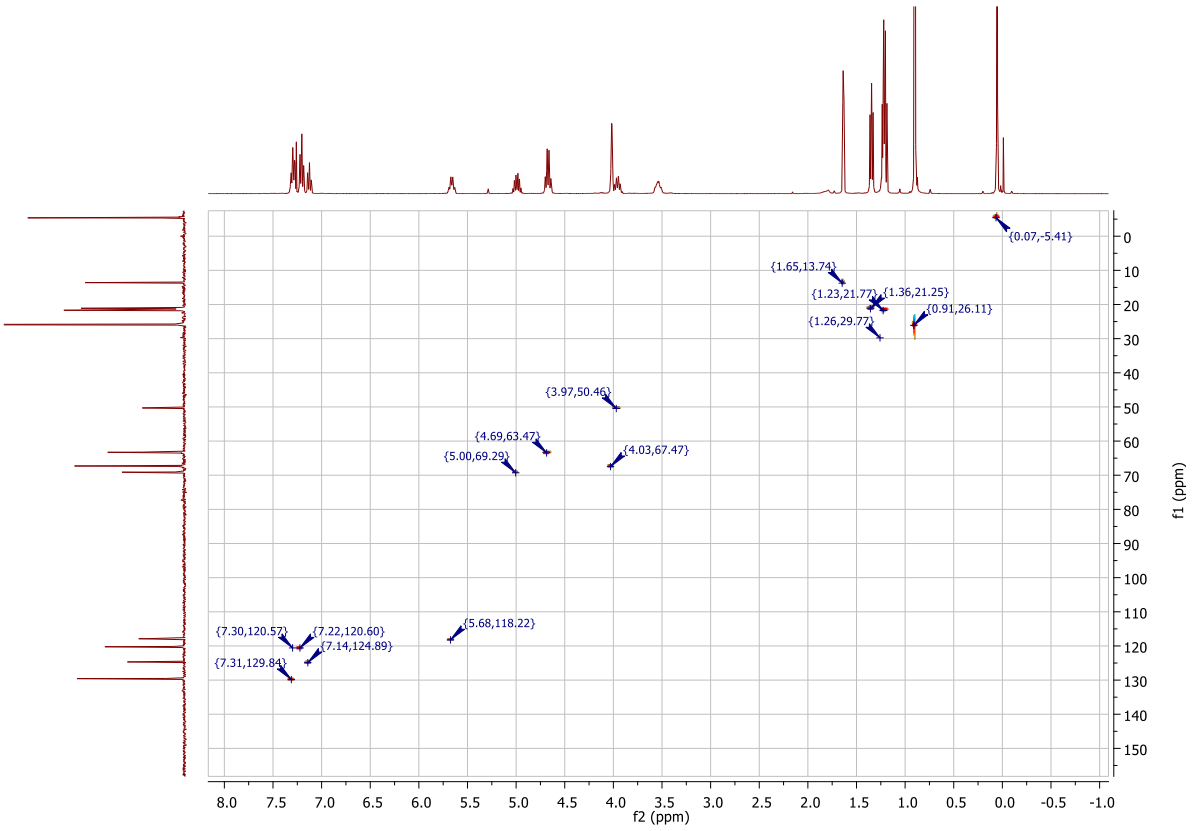
^{1}H COSY



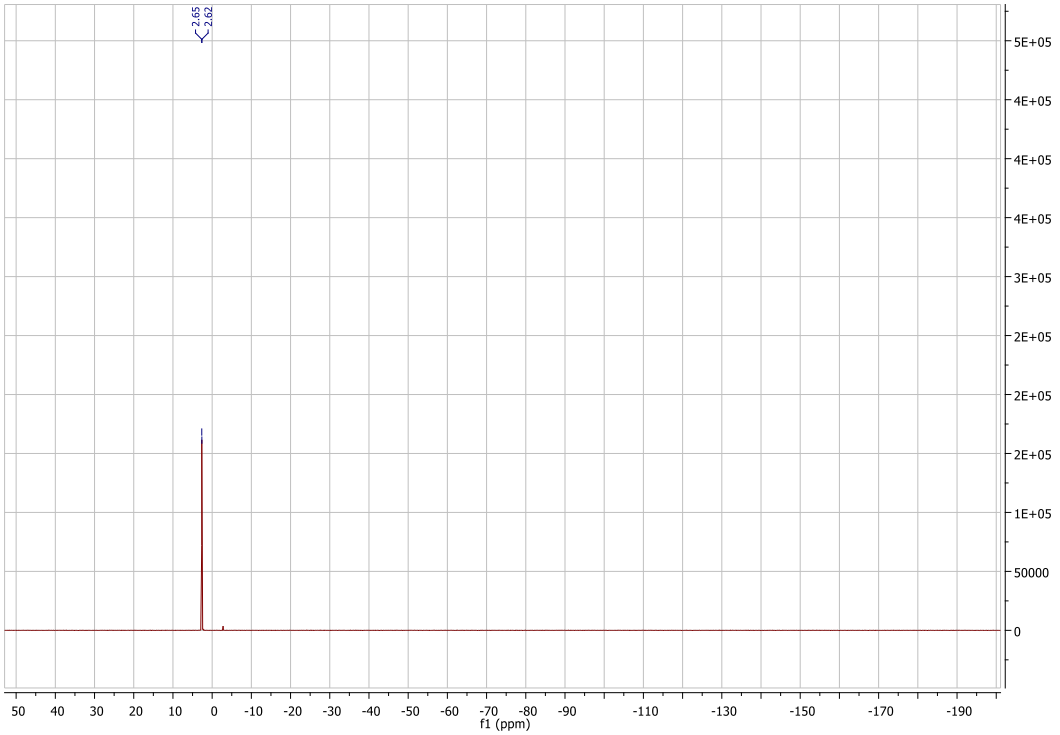
^{13}C DEPT-45



HSQC

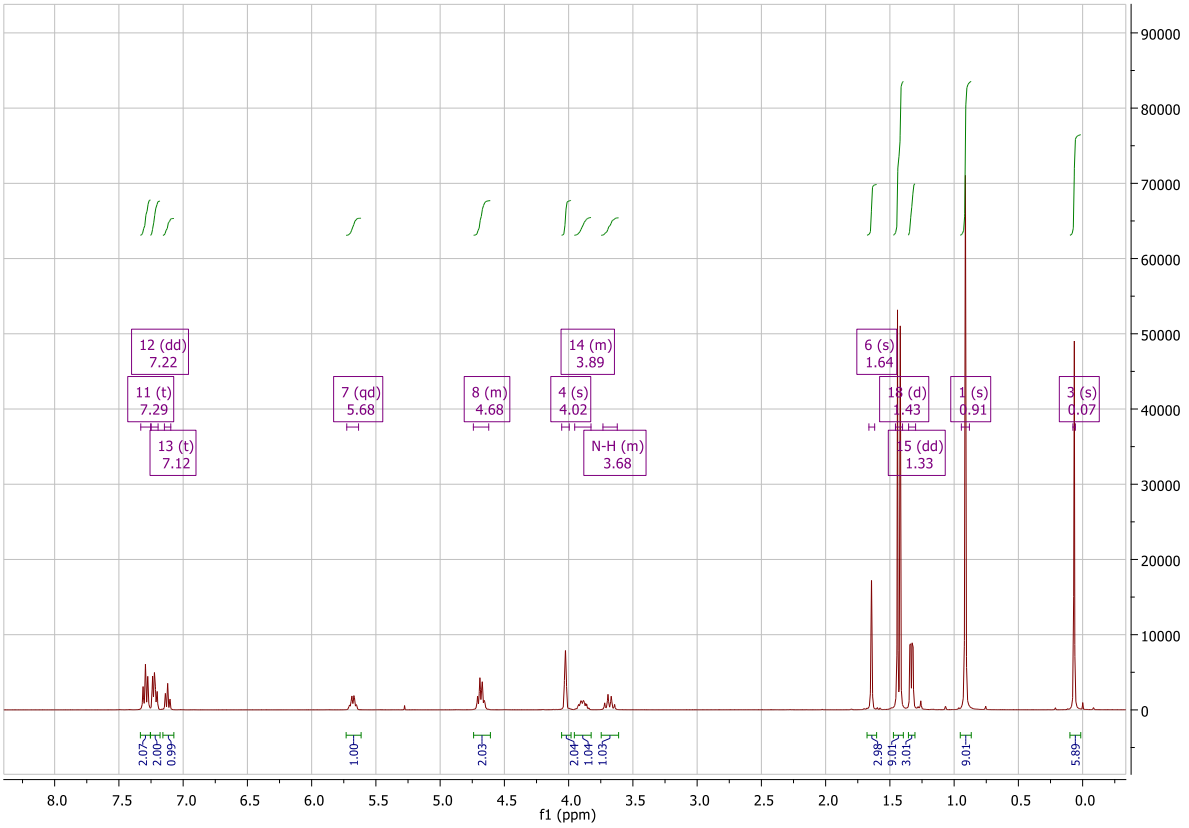

{0.07,-5.41}
{1.65,13.74}
{1.23,21.77}
{1.36,21.25}
{0.91,26.11}
{1.26,29.77}
{3.97,50.46}
{4.69,63.47}
{5.00,69.29}
{4.03,67.47}
{7.30,120.57}
{7.22,120.60}
{7.14,124.89}
{7.31,129.84}
{5.68,118.22}
f2 (ppm)
f1 (ppm)

^{31}P NMR

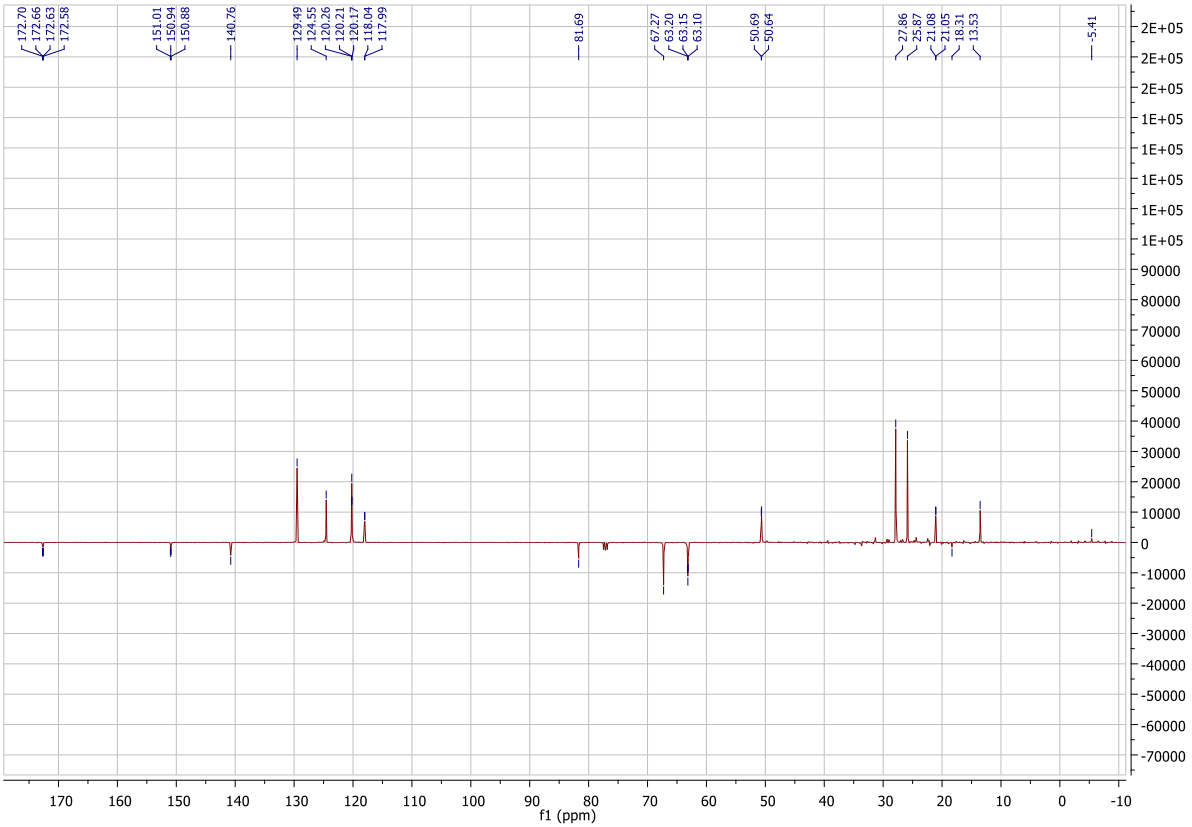

2.65
2.62
f1 (ppm)

Tert-butyl ((((E)-4-((tert-butyldimethylsilyl)oxy)-3-methylbut-2-en-1-yl)oxy)(phenoxy)phosphoryl)-L-alaninate, 5d:

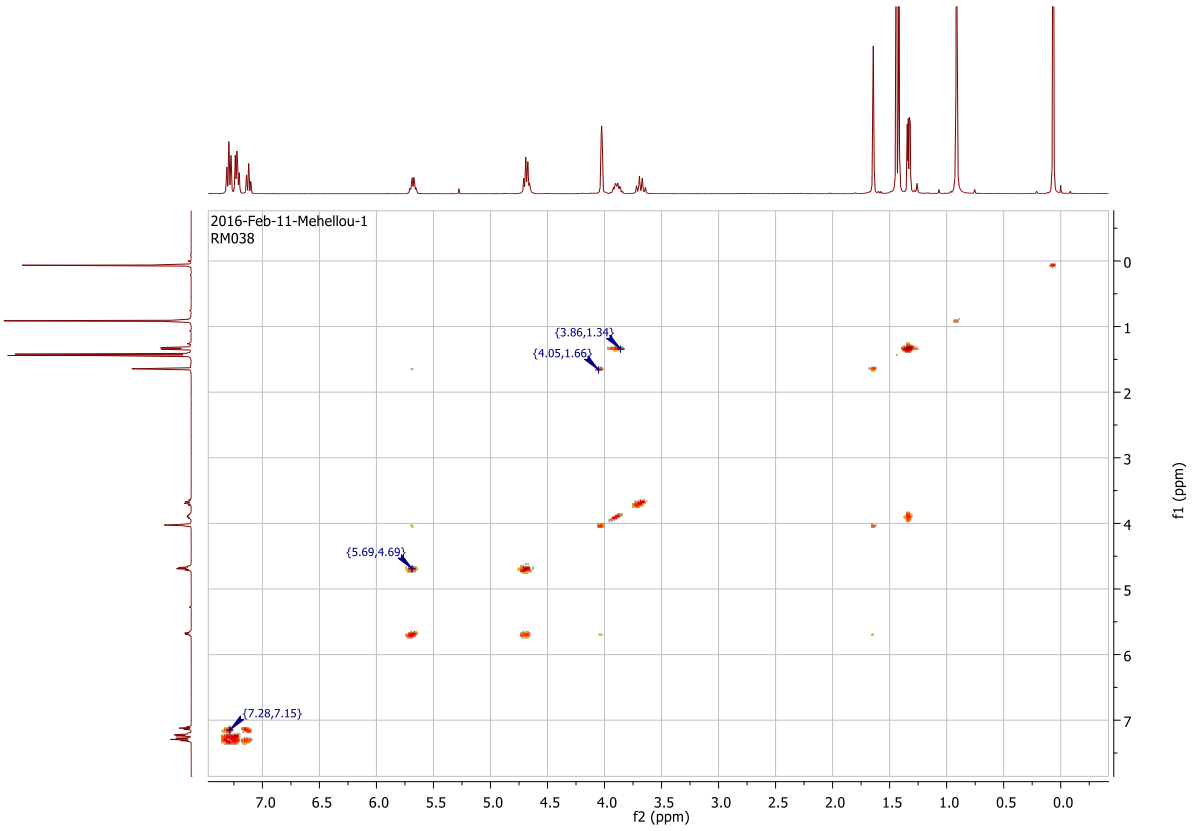
^{1}H NMR



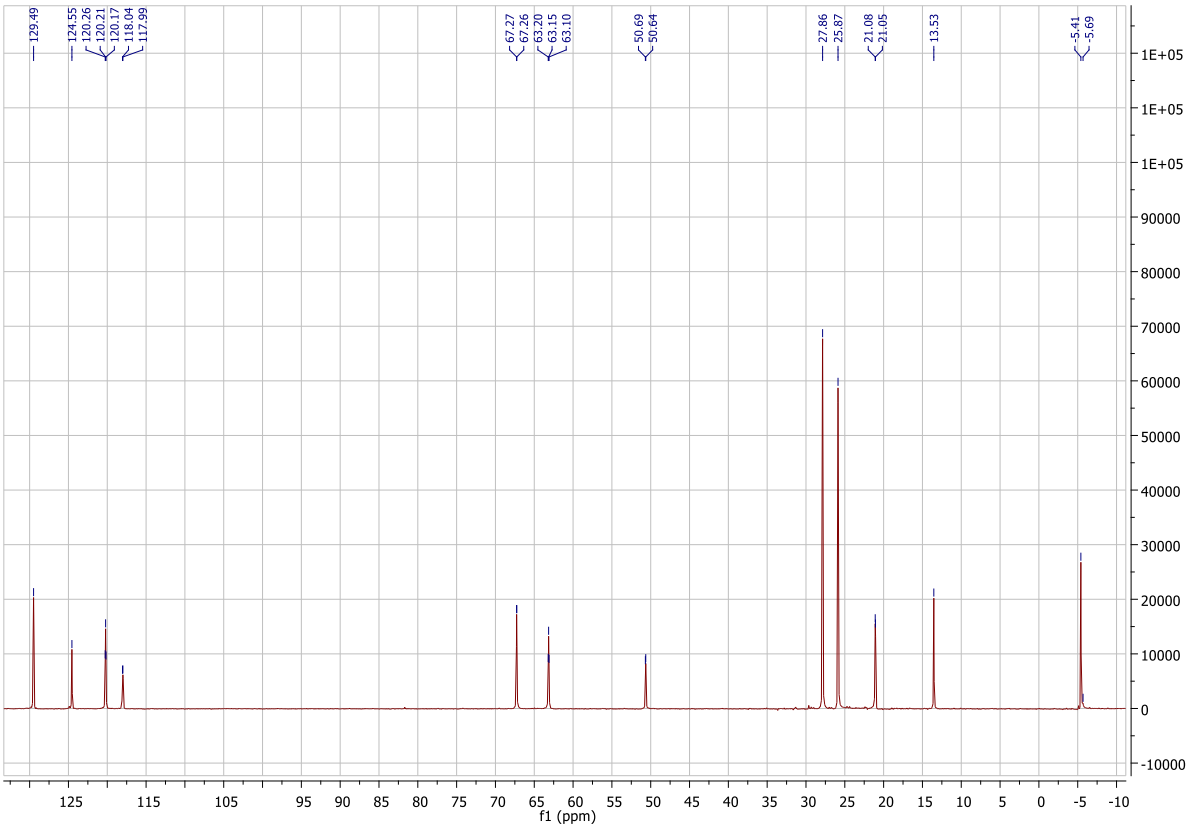
^{13}C NMR



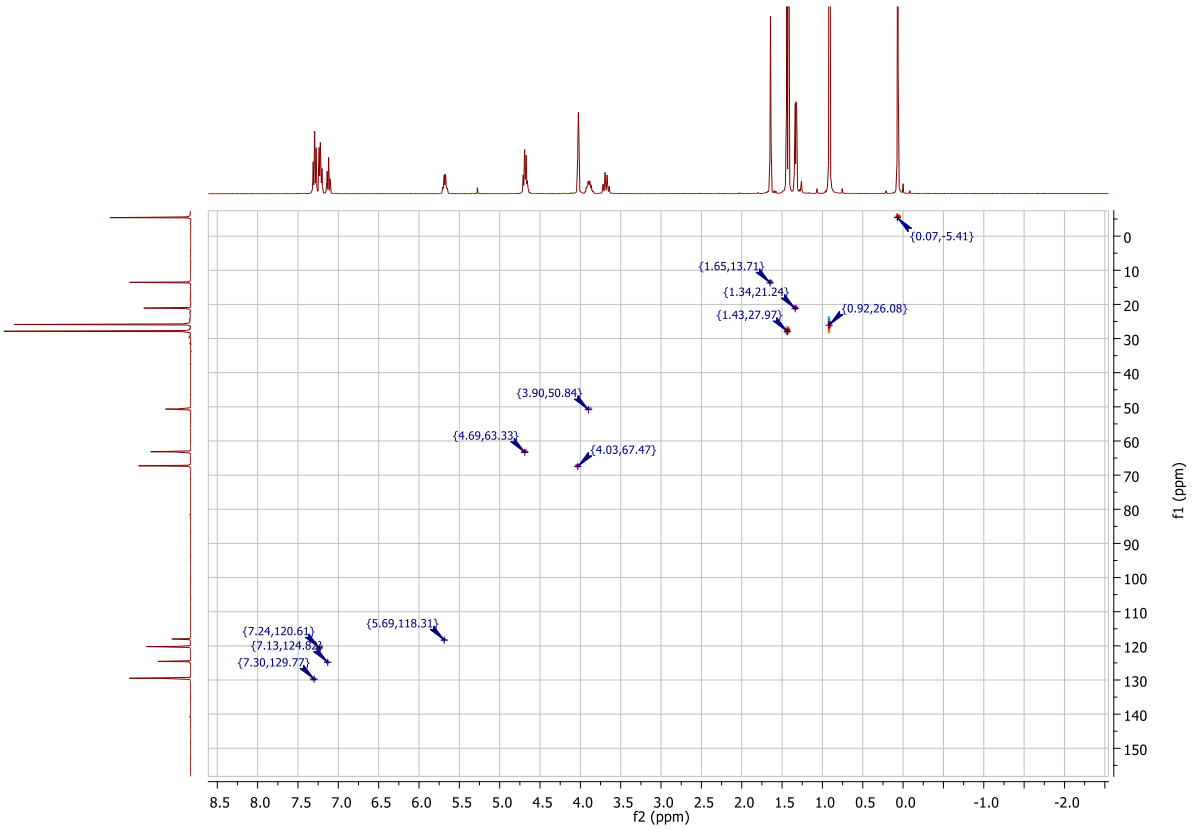
^{1}H COSY



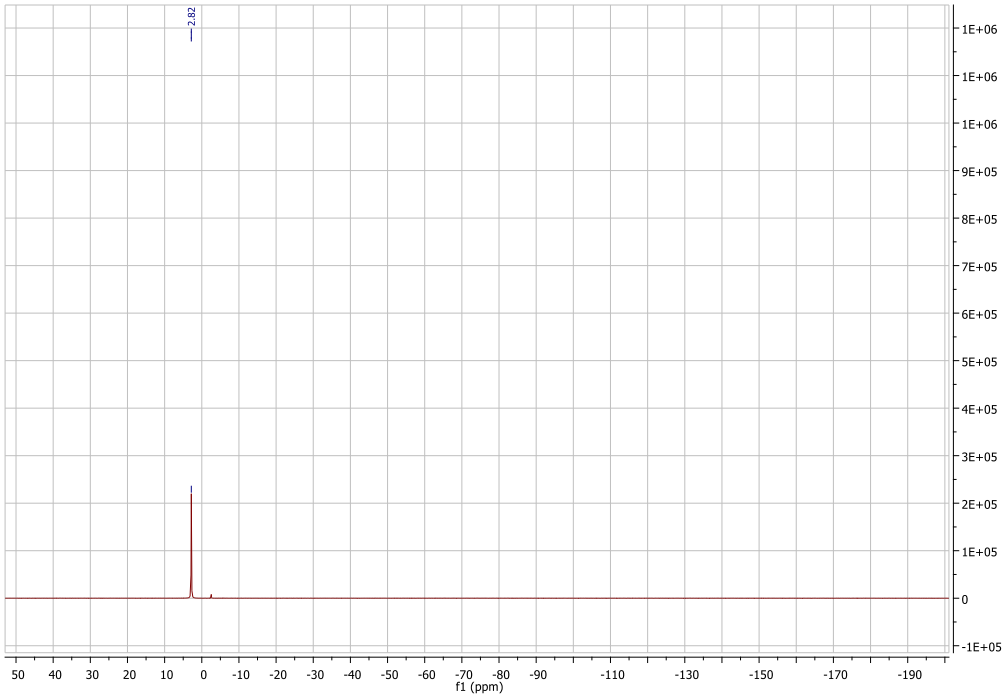
^{13}C DEPT-45



HSQC

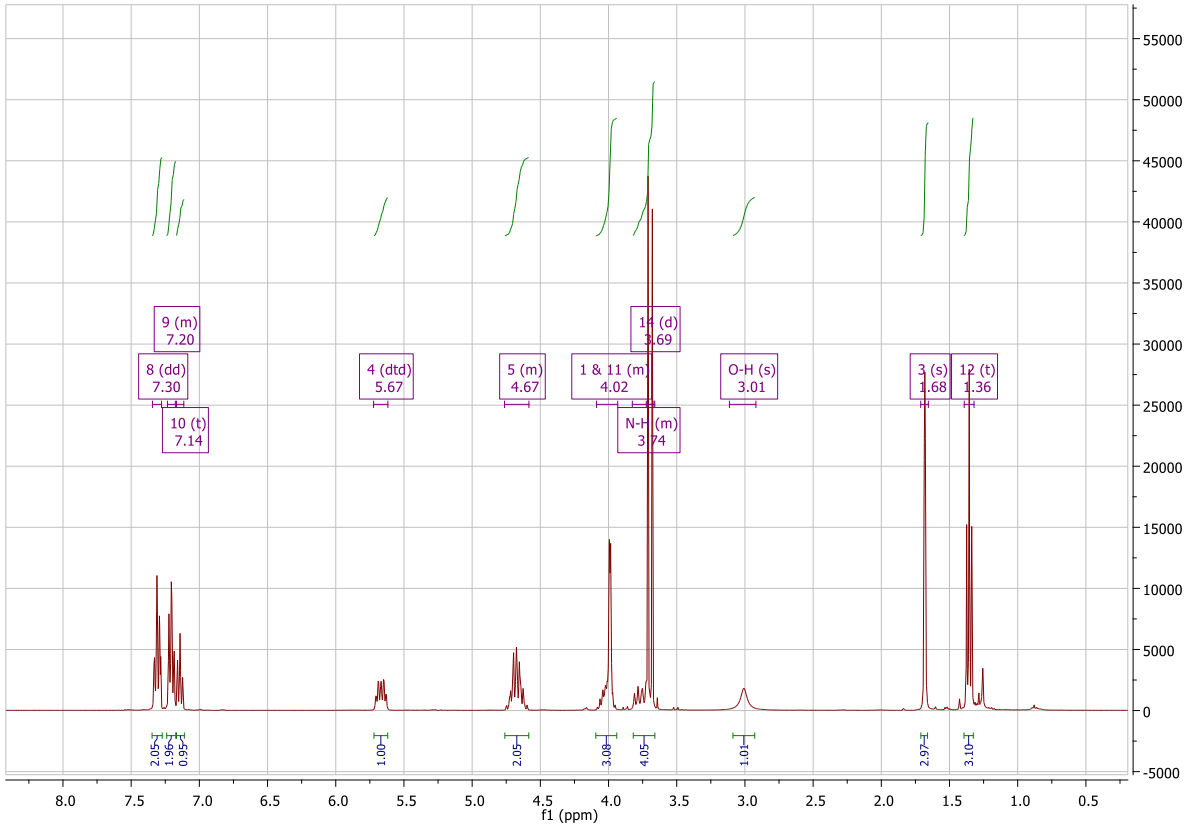


^{31}P NMR

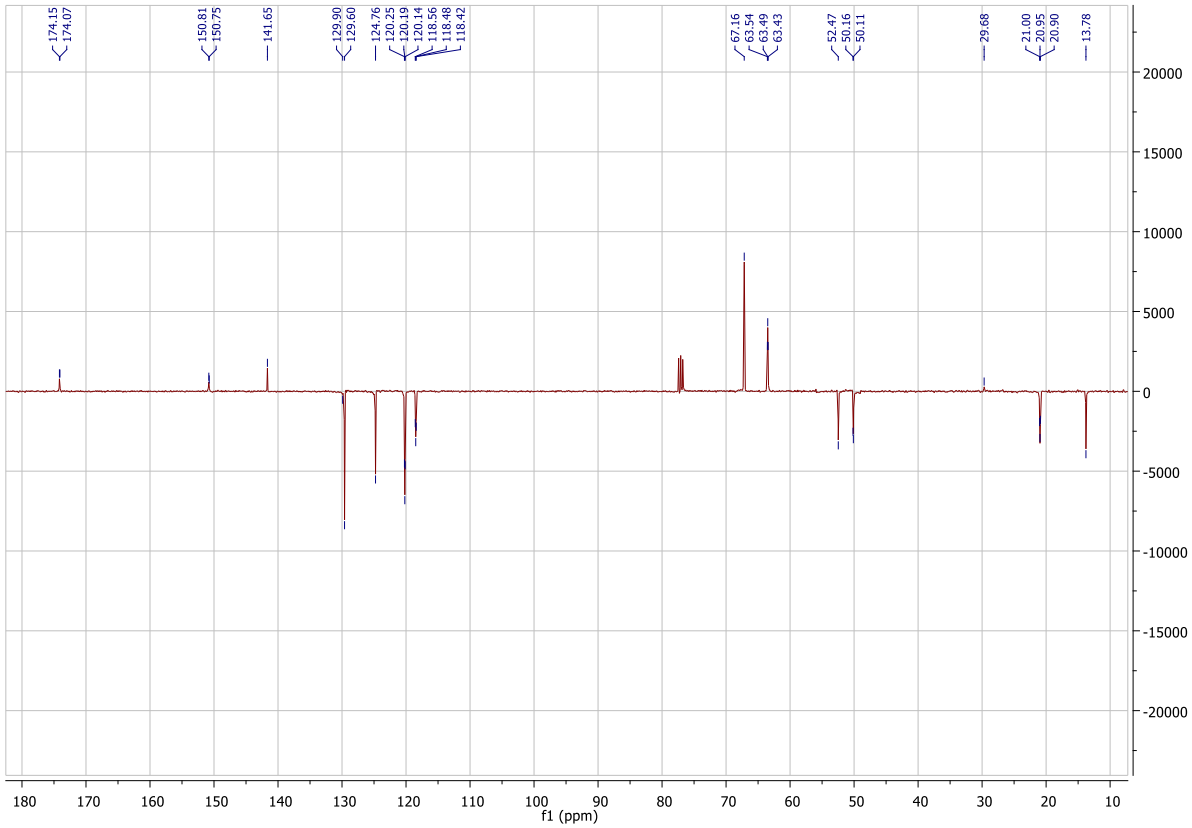


Methyl ((((E)-4-hydroxy-3-methylbut-2-en-1-yl)oxy)(phenoxy)phosphoryl)-L-alaninate, 6a:

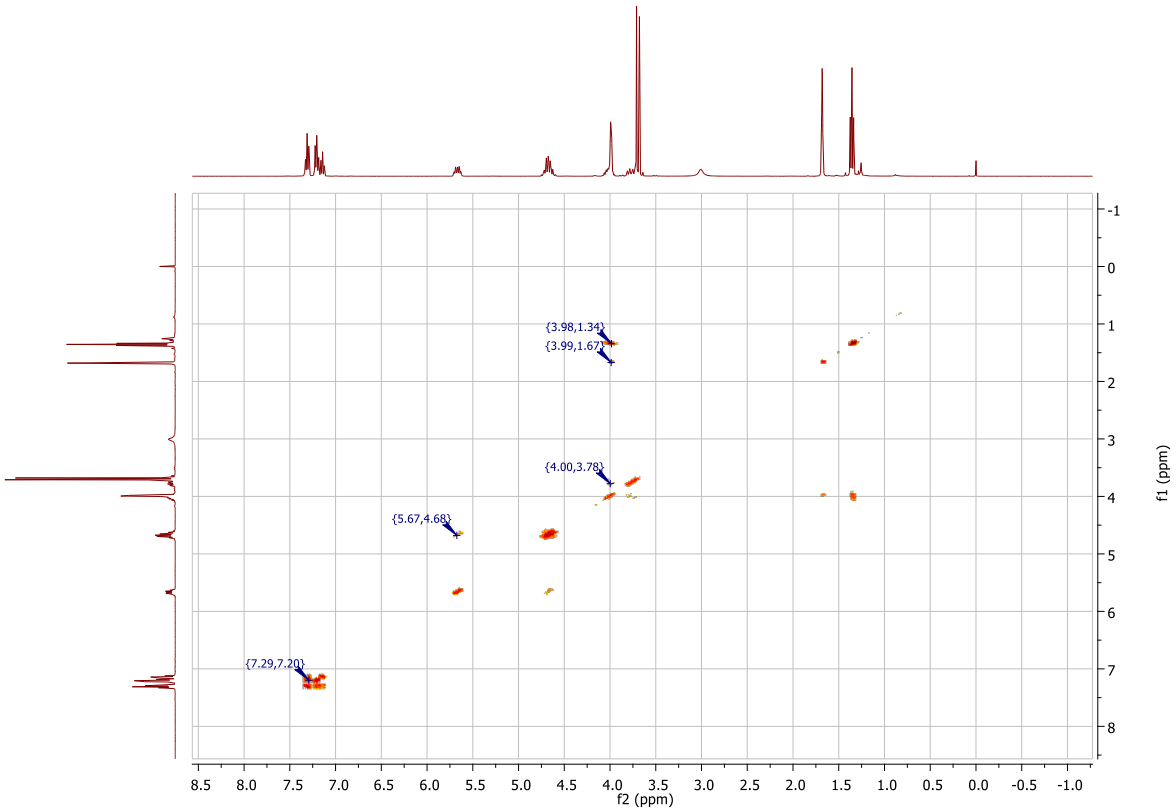
1H NMR



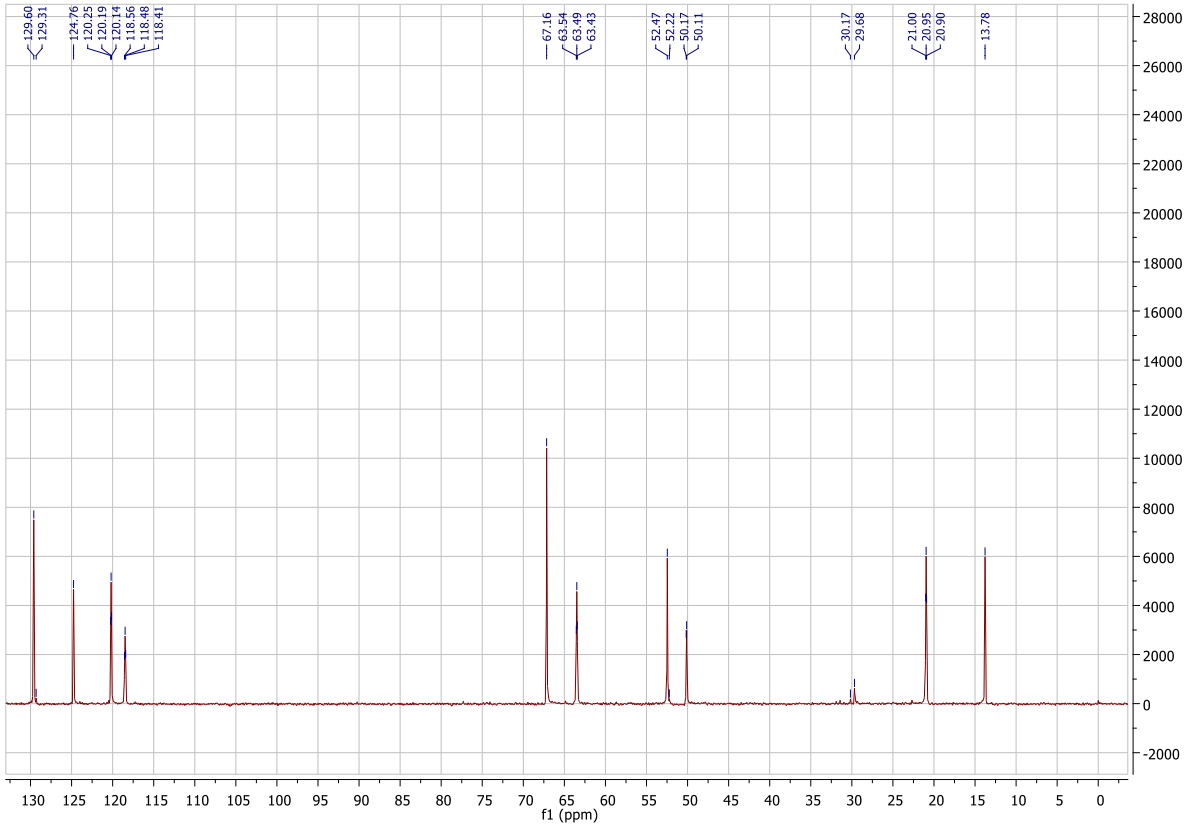
^{13}C NMR



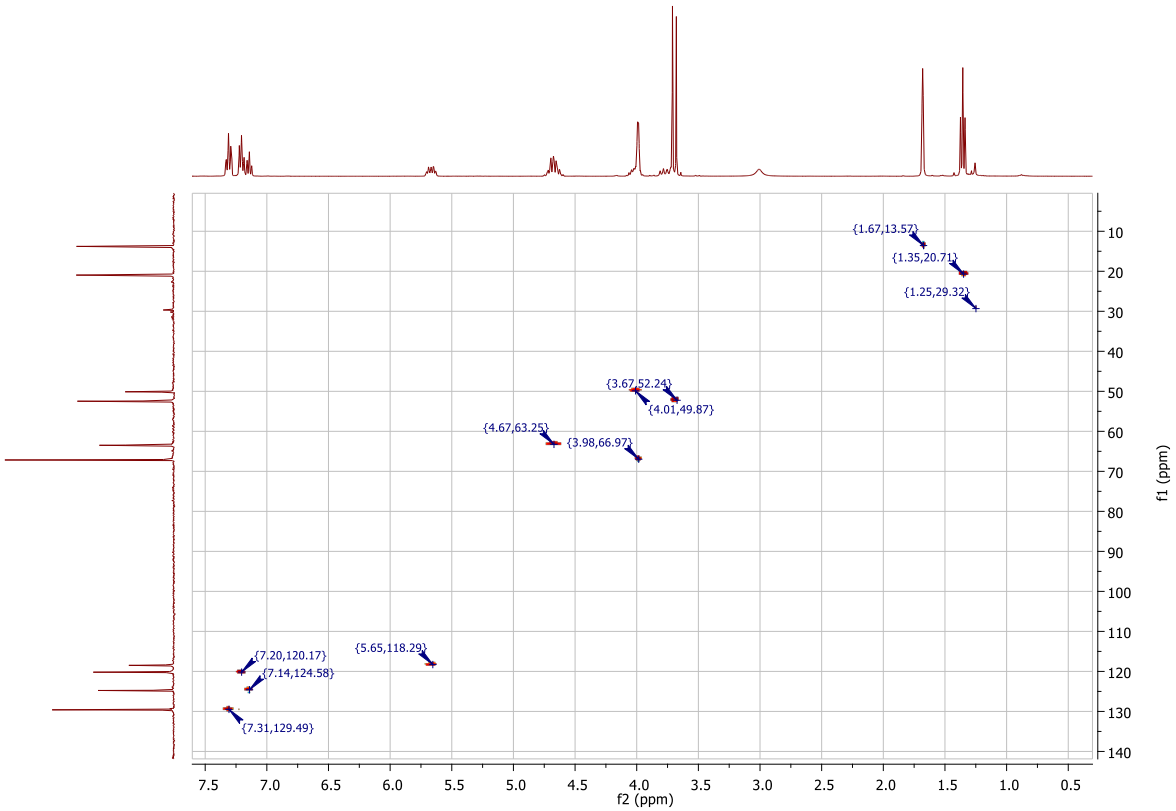
^{1}H COSY



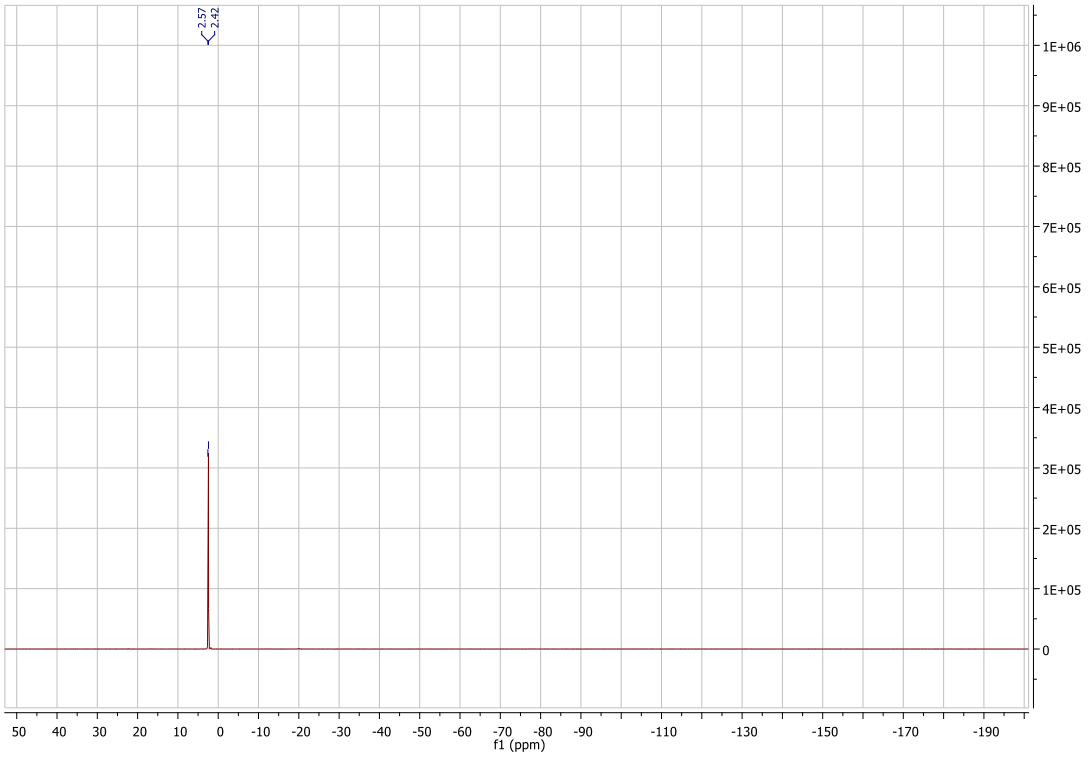
`^{13}C DEPT-45



HSQC

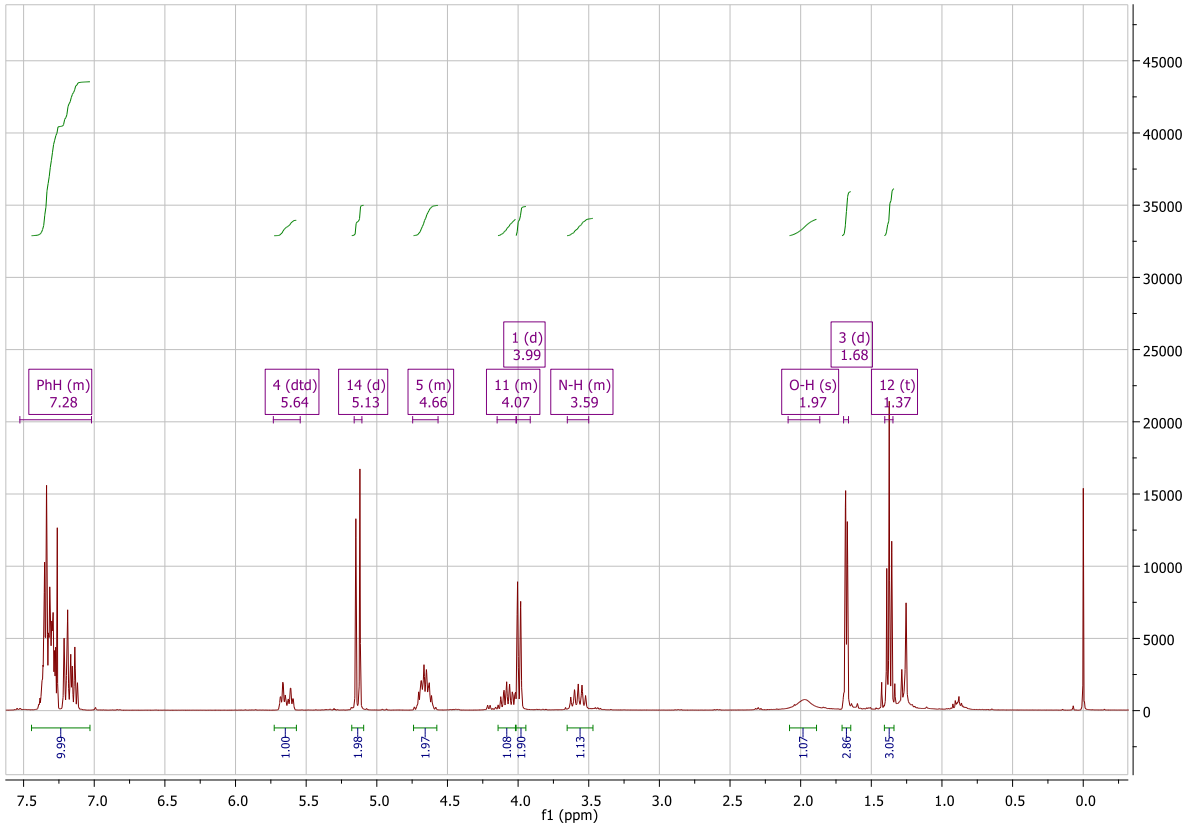


^{31}P NMR

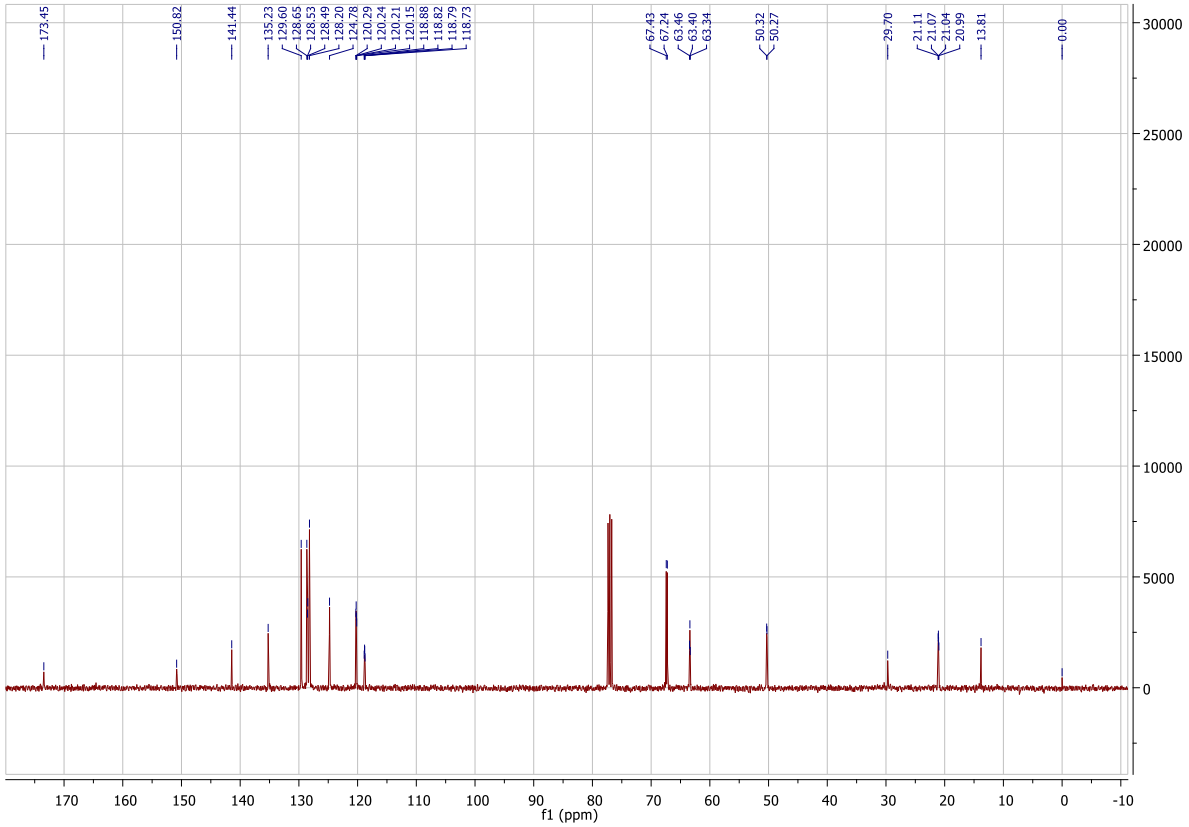


Benzyl ((((E)-4-hydroxy-3-methylbut-2-en-1-yl)oxy)(phenoxy)phosphoryl)-L-alaninate, 6b:

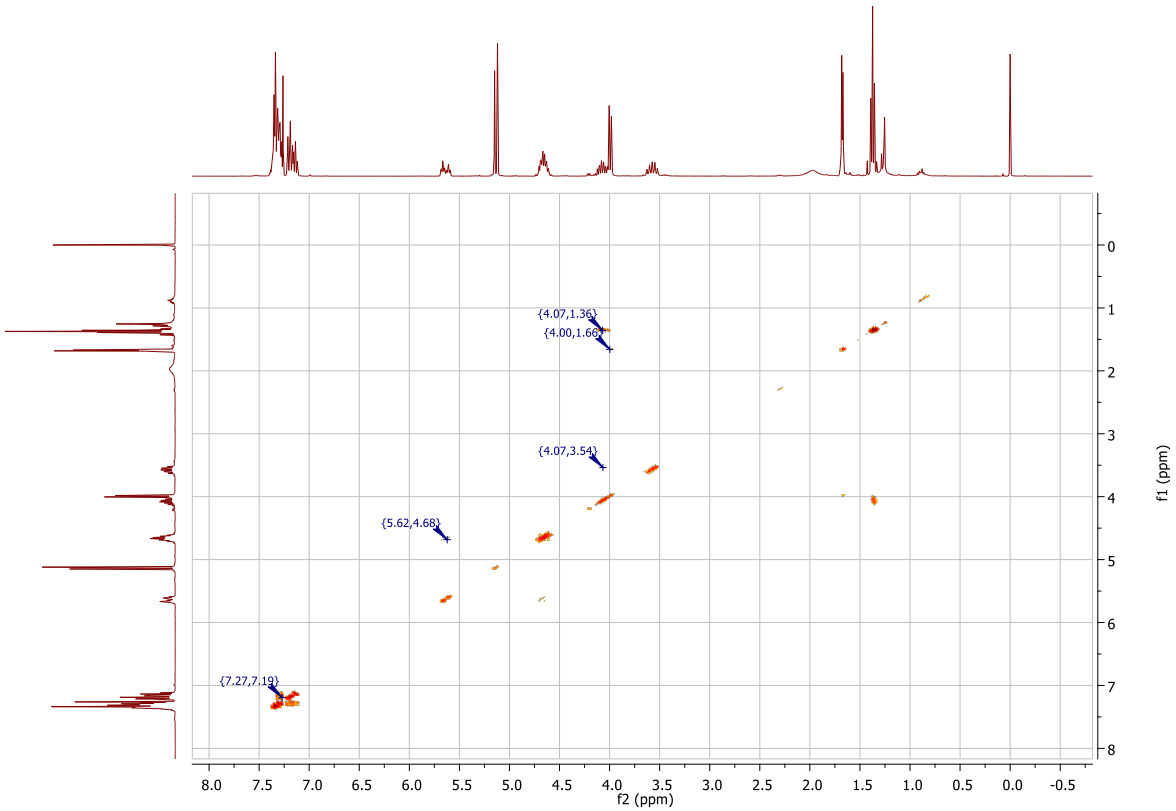
^{1}H NMR



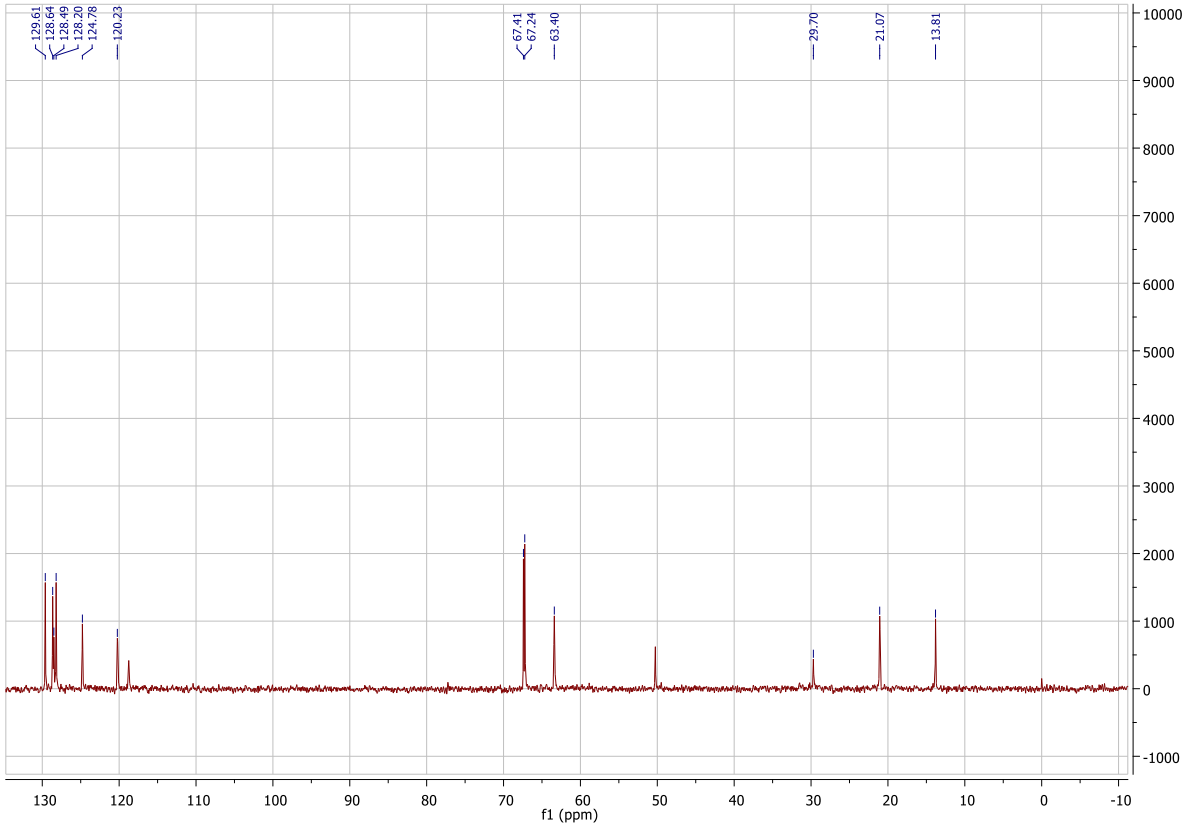
^{13}C NMR



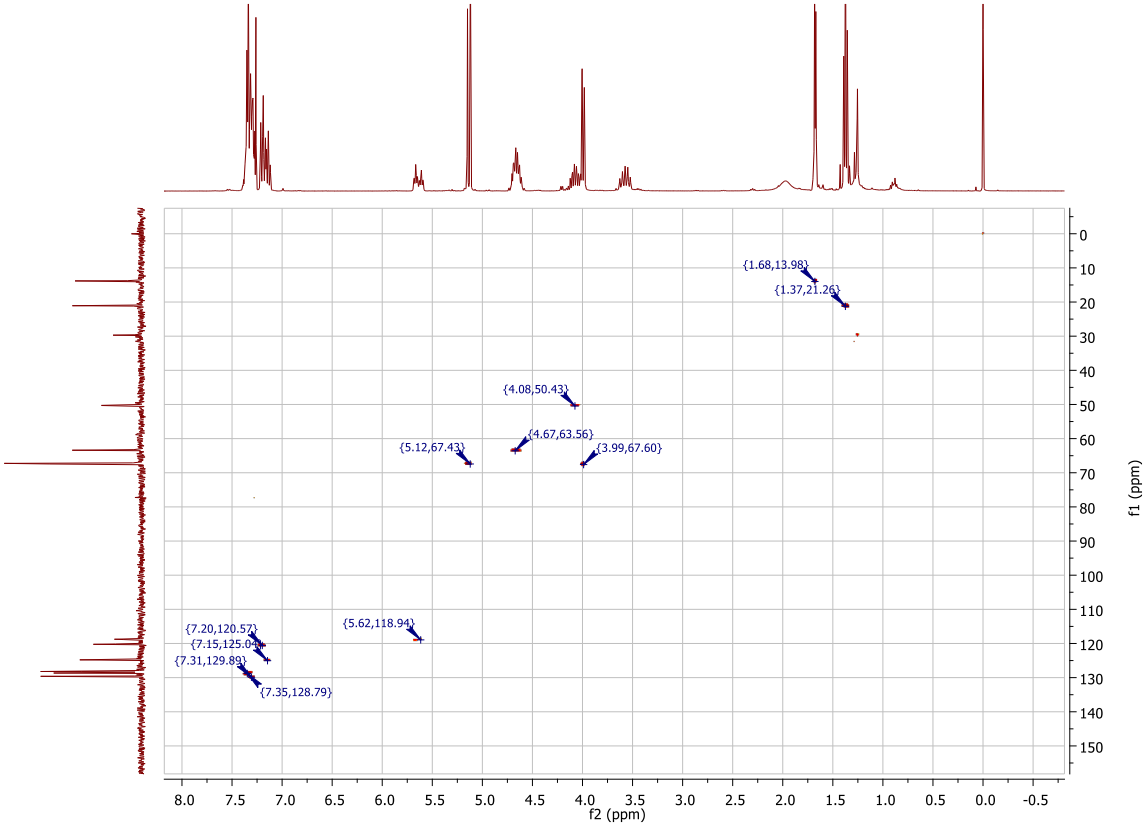
^{1}H COSY



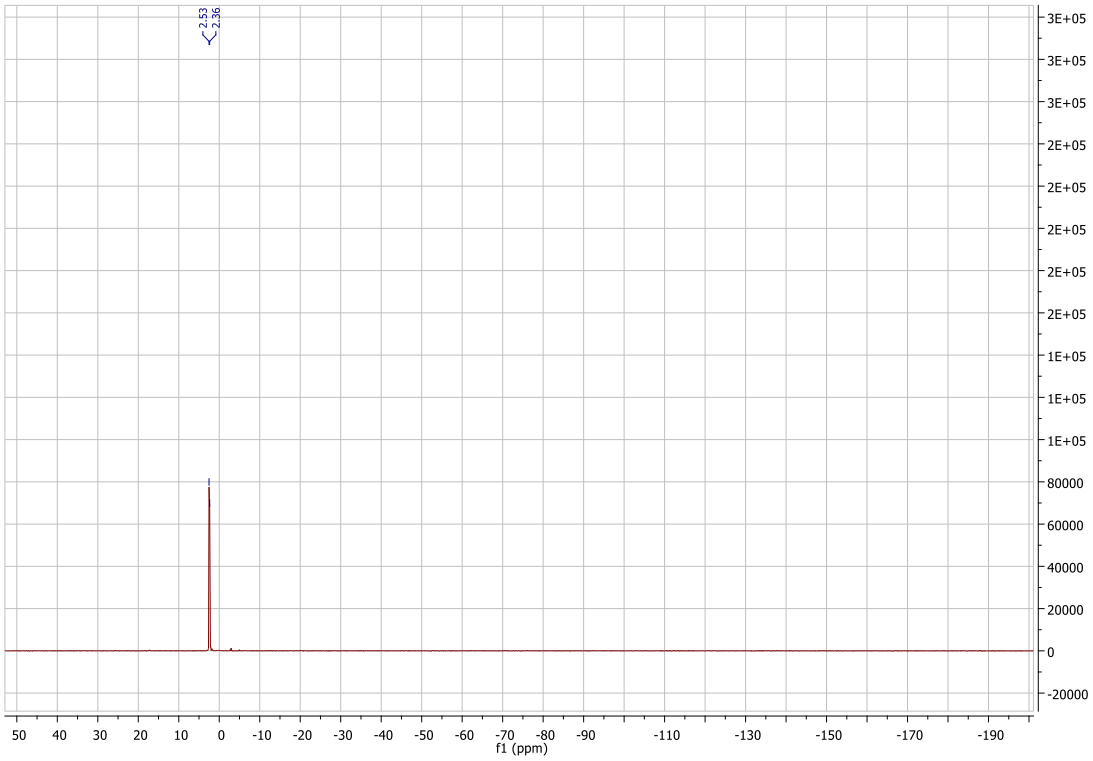
^{13}C DEPT-45



HSQC

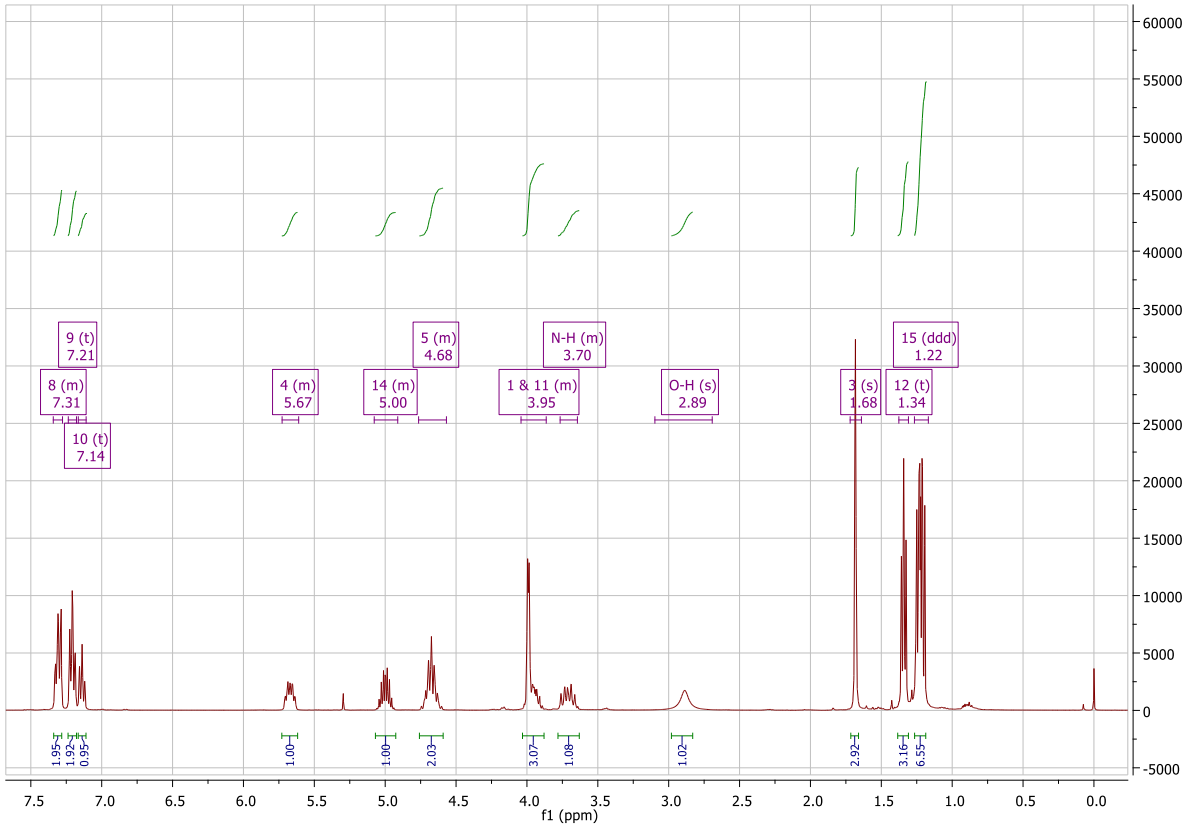


^{31}P NMR

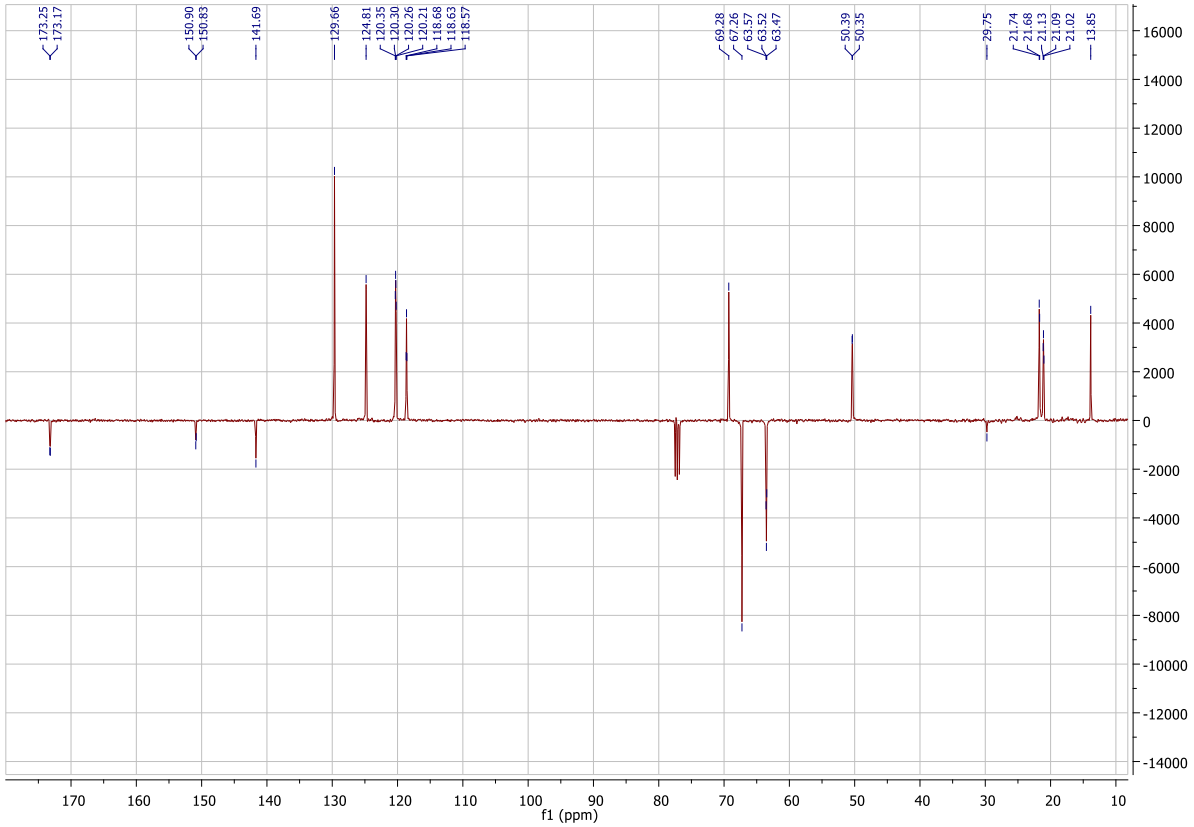


Isopropyl ((((E)-4-hydroxy-3-methylbut-2-en-1-yl)oxy)(phenoxy)phosphoryl)-L-alaninate, 6c:

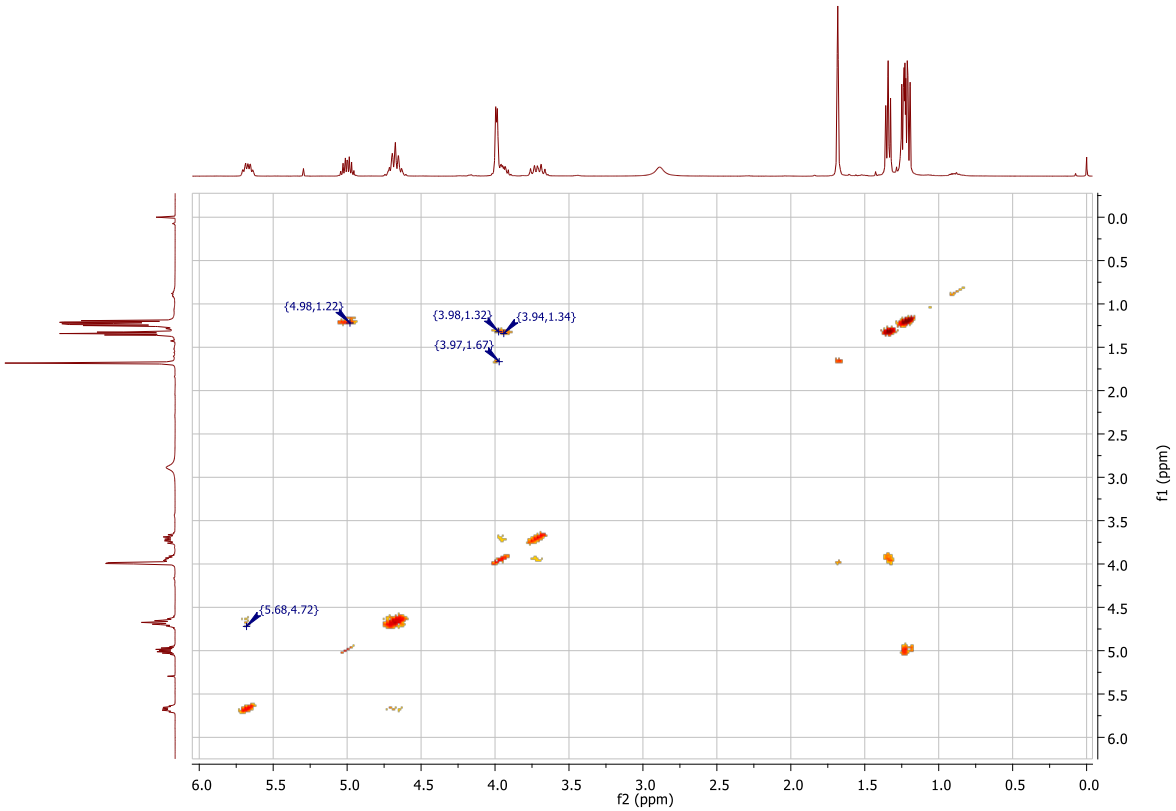
^{1}H NMR



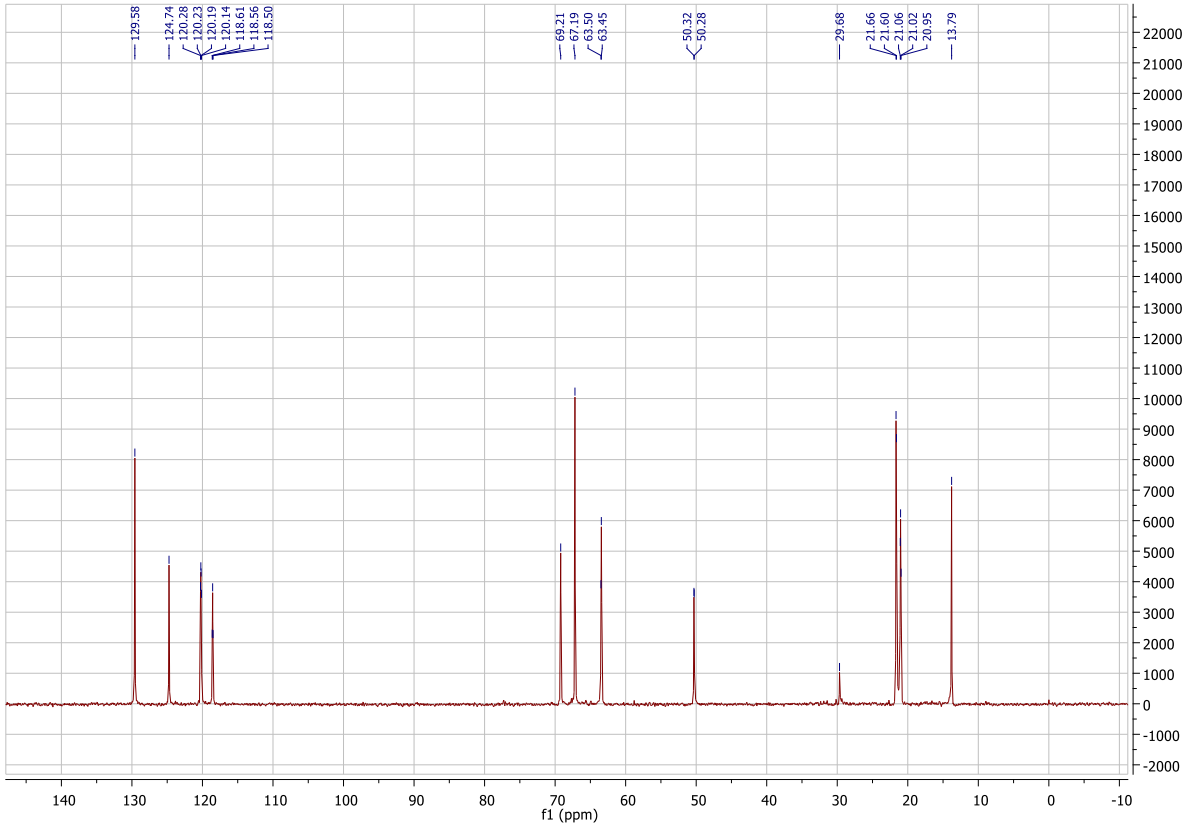
^{13}C NMR



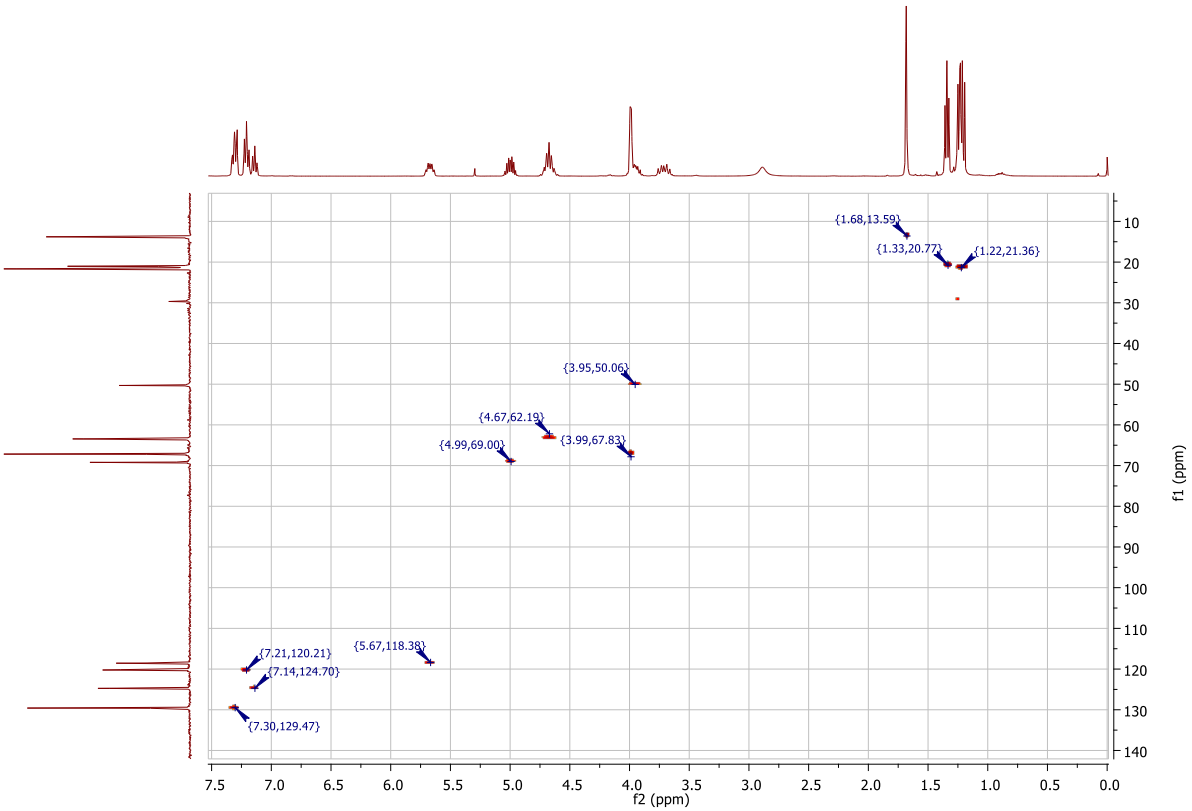
^{1}H COSY



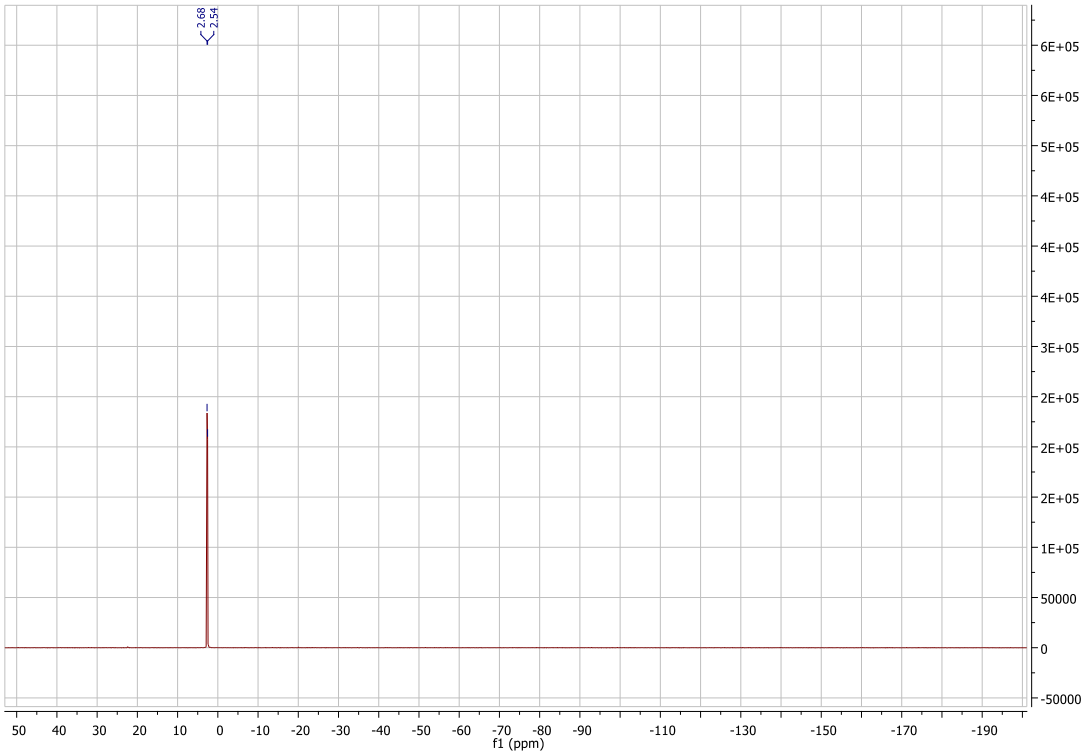
^{13}C DEPT-45



HSQC

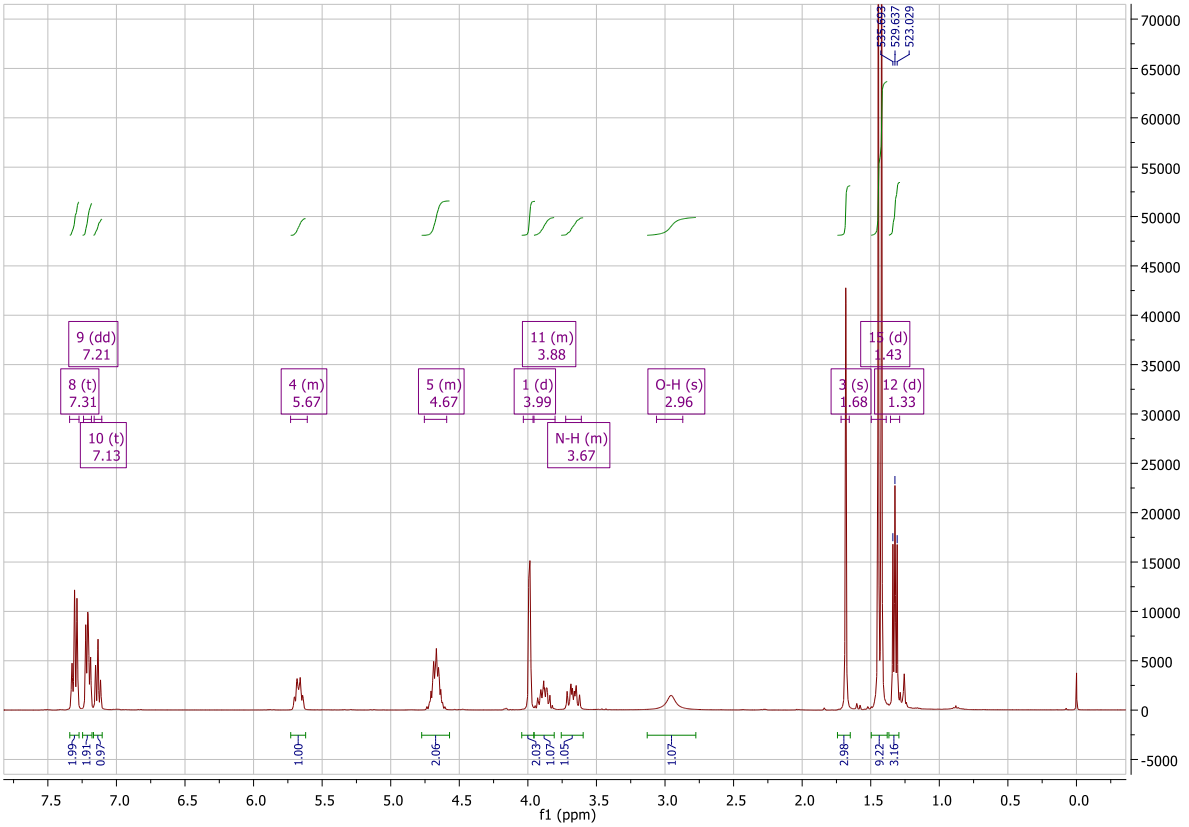

{1.68,13.59}
{1.33,20.77}
{1.22,21.36}
{3.95,50.06}
{4.67,62.19}
{4.99,69.00}
{3.99,67.83}
{5.67,118.38}
{7.21,120.21}
{7.14,124.70}
{7.30,129.47}
f1 (ppm)
f2 (ppm)

^{31}P NMR

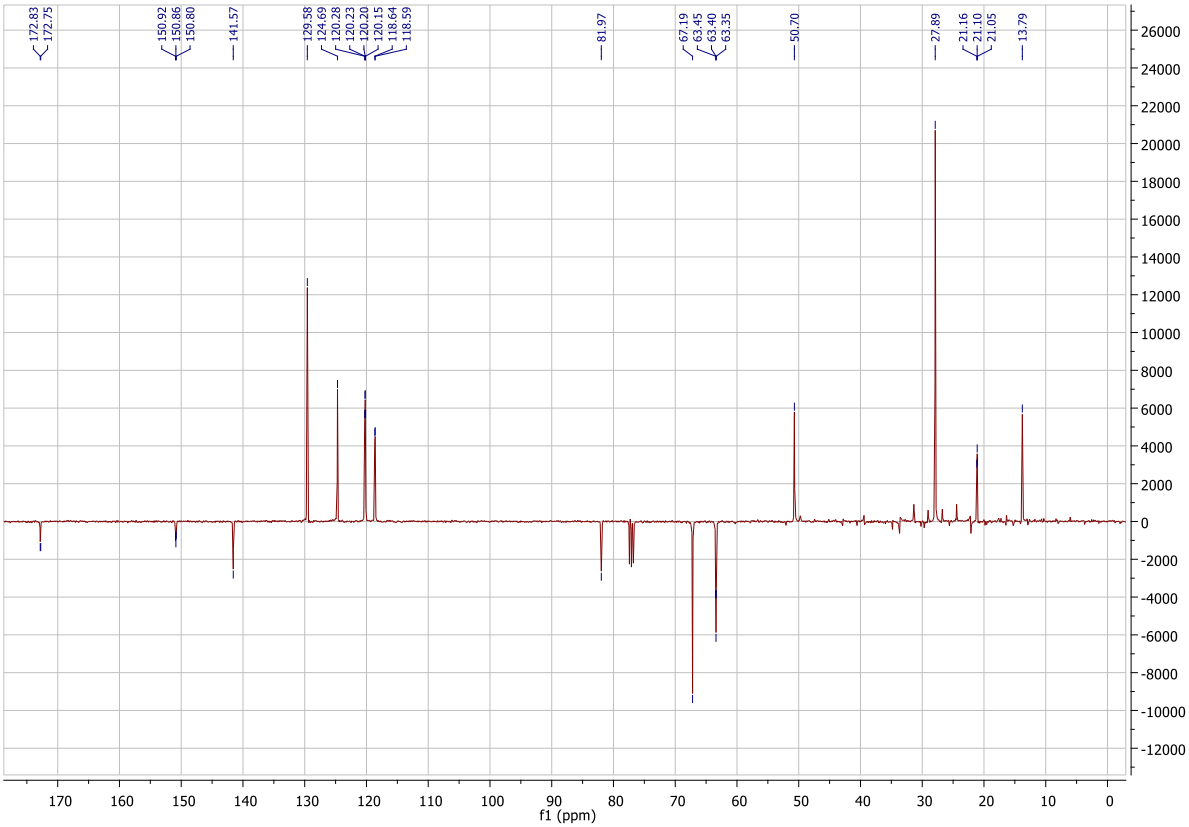

2.68
2.54
f1 (ppm)

Tert-butyl ((((E)-4-hydroxy-3-methylbut-2-en-1-yl)oxy)(phenoxy)phosphoryl)-L-alaninate, 6d:

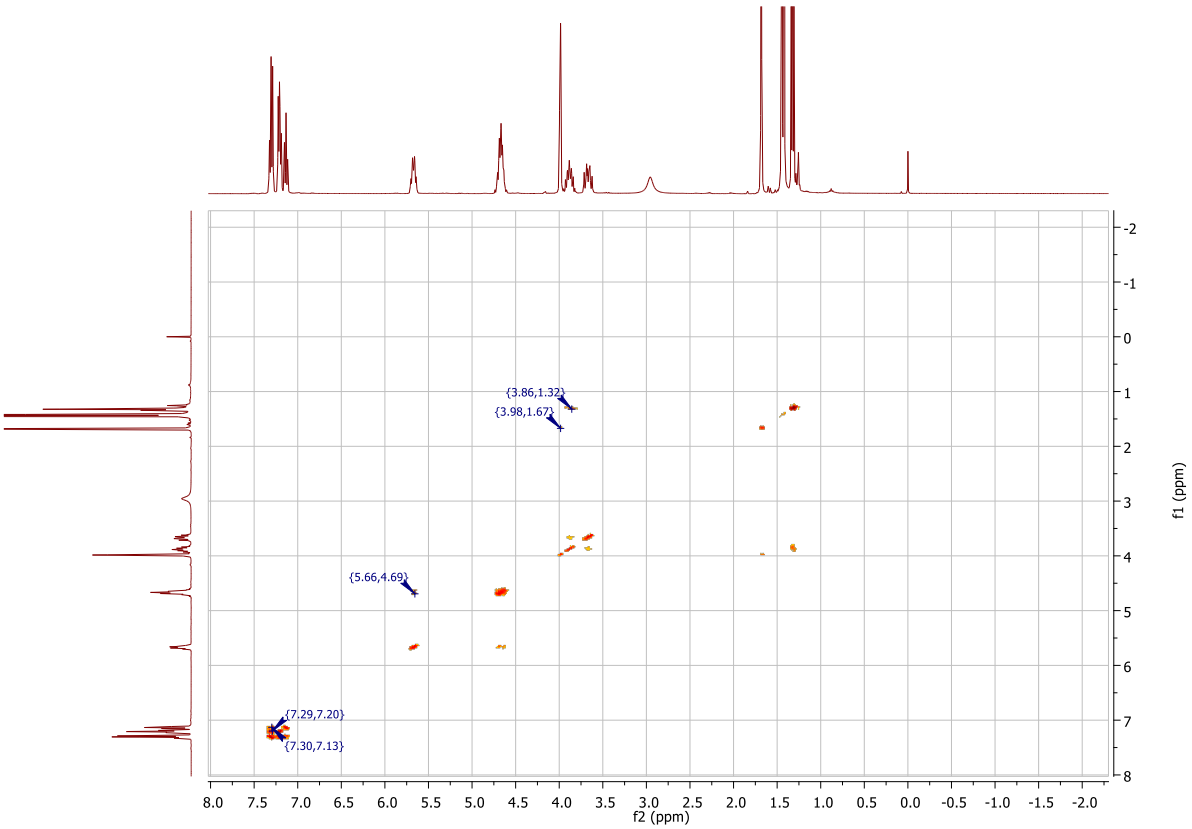
^{1}H NMR



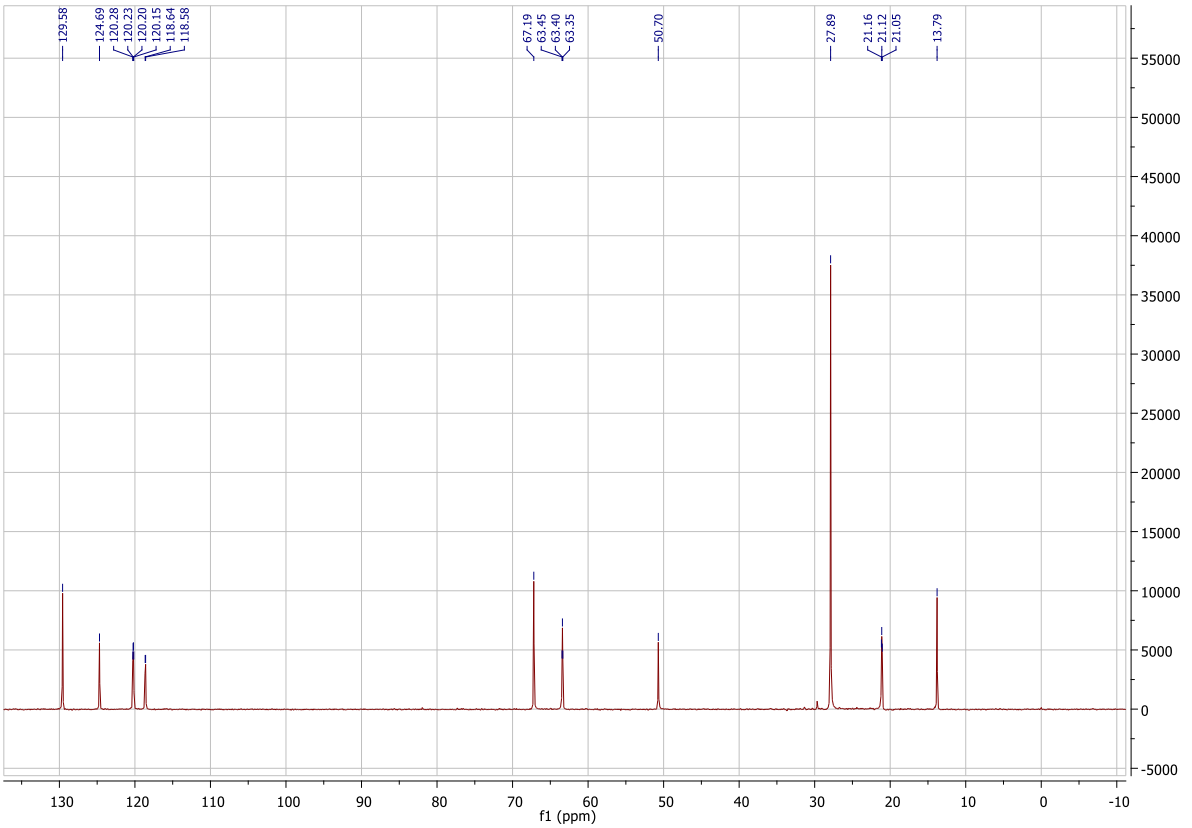
^{13}C NMR



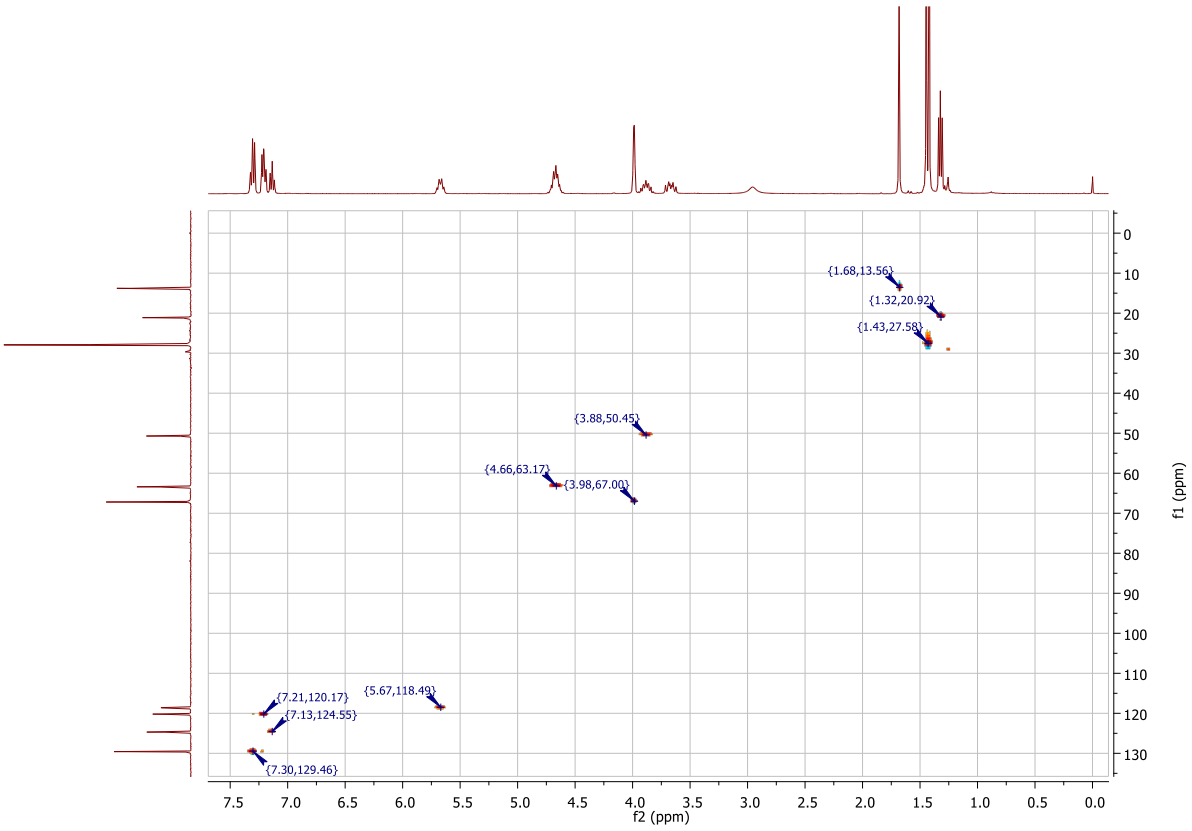
^{1}H COSY



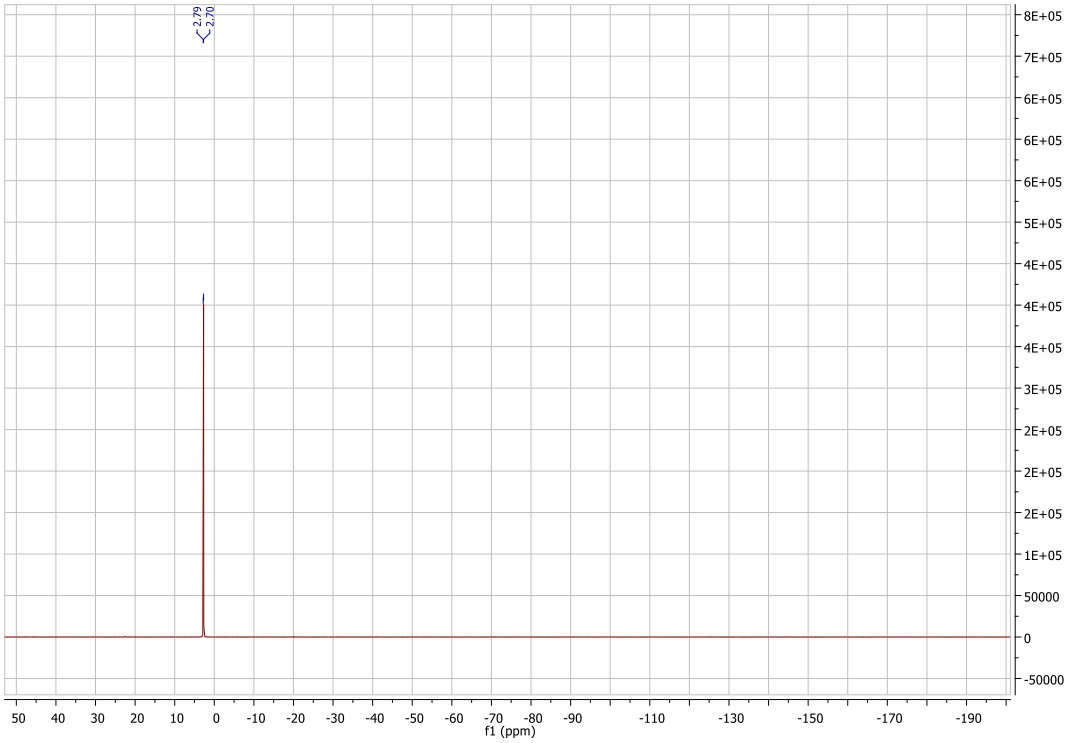
^{13}C DEPT-45



HSQC


{1.68,13.56}
{1.32,20.92}
{1.43,27.58}
{3.88,50.45}
{4.66,63.17}
{3.98,67.00}
{7.21,120.17}
{5.67,118.49}
{7.13,124.55}
{7.30,129.46}
f2 (ppm)
f1 (ppm)

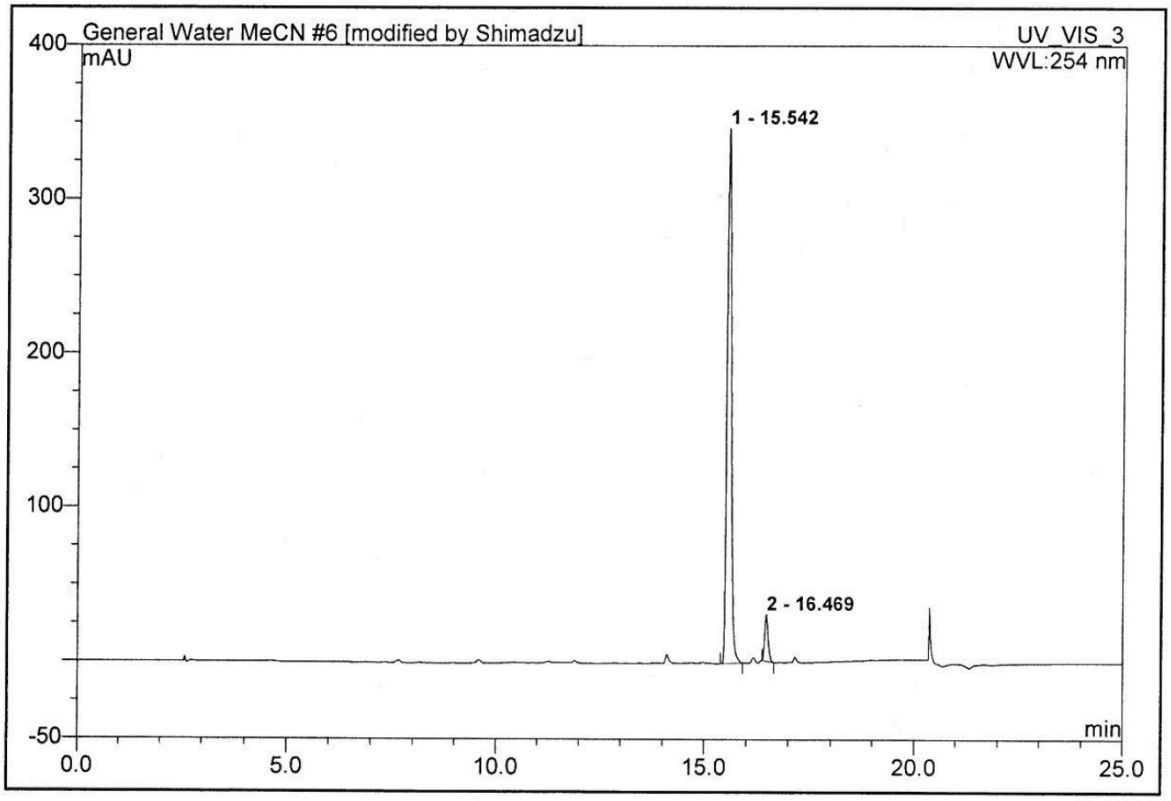
^{31}P NMR


2.79
2.70
f1 (ppm)

III. HPLC spectra

6 RM27 Compound 5a

Sample Name:	**RM27**	*Injection Volume:*	**20.0**
Vial Number:	**1_4**	*Channel:*	**UV_VIS_3**
Sample Type:	**unknown**	*Wavelength:*	**254**
Control Program:	**MeCN 25min gradient**	*Bandwidth:*	**8**
Quantif. Method:	**General Method**	*Dilution Factor:*	**1.0000**
Recording Time:	**24/2/2017 13:05**	*Sample Weight:*	**1.0000**
Run Time (min):	**25.00**	*Sample Amount:*	**1.0000**

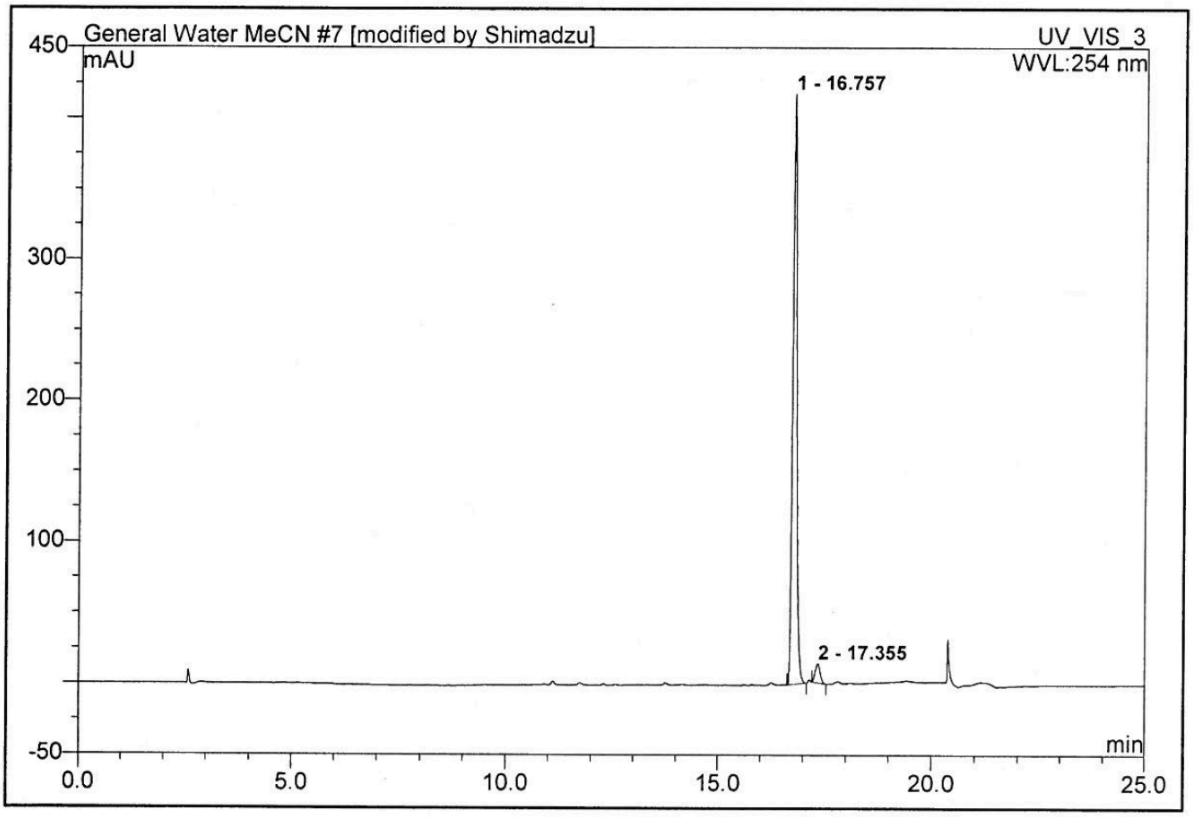


No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	15.54	n.a.	347.176	42.070	94.04	n.a.	BMB
2	16.47	n.a.	30.133	2.667	5.96	n.a.	BMB
Total:			377.309	44.737	100.00	0.000	

7 RM29

Compound 5b

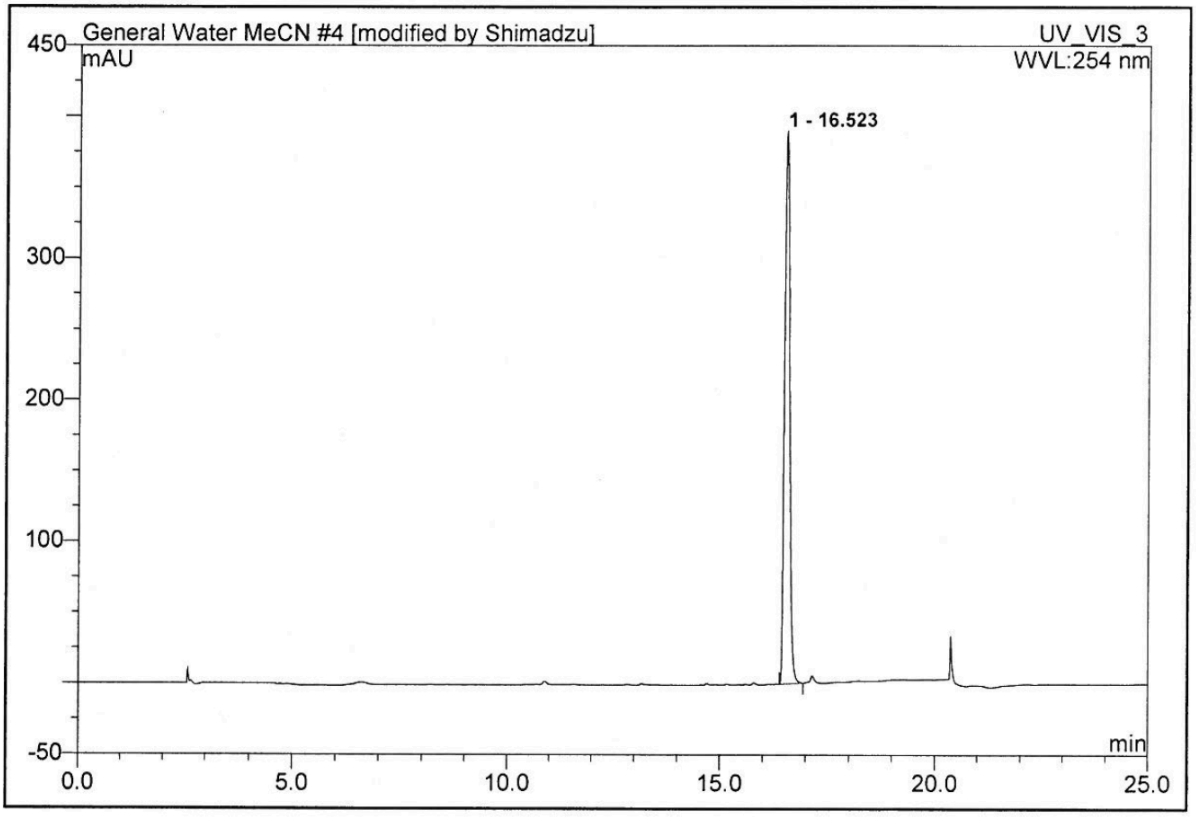
Sample Name:	**RM29**	*Injection Volume:*	**20.0**
Vial Number:	**1_5**	*Channel:*	**UV_VIS_3**
Sample Type:	**unknown**	*Wavelength:*	**254**
Control Program:	**MeCN 25min gradient**	*Bandwidth:*	**8**
Quantif. Method:	**General Method**	*Dilution Factor:*	**1.0000**
Recording Time:	**24/2/2017 13:30**	*Sample Weight:*	**1.0000**
Run Time (min):	**25.00**	*Sample Amount:*	**1.0000**



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	16.76	n.a.	417.330	47.534	96.47	n.a.	BMB
2	17.35	n.a.	13.383	1.742	3.53	n.a.	BMB
Total:			430.713	49.275	100.00	0.000	

4 RM20 Compound 5c

Sample Name:	**RM20**	*Injection Volume:*	**20.0**
Vial Number:	**1_2**	*Channel:*	**UV_VIS_3**
Sample Type:	**unknown**	*Wavelength:*	**254**
Control Program:	**MeCN 25min gradient**	*Bandwidth:*	**8**
Quantif. Method:	**General Method**	*Dilution Factor:*	**1.0000**
Recording Time:	**24/2/2017 12:14**	*Sample Weight:*	**1.0000**
Run Time (min):	**25.00**	*Sample Amount:*	**1.0000**

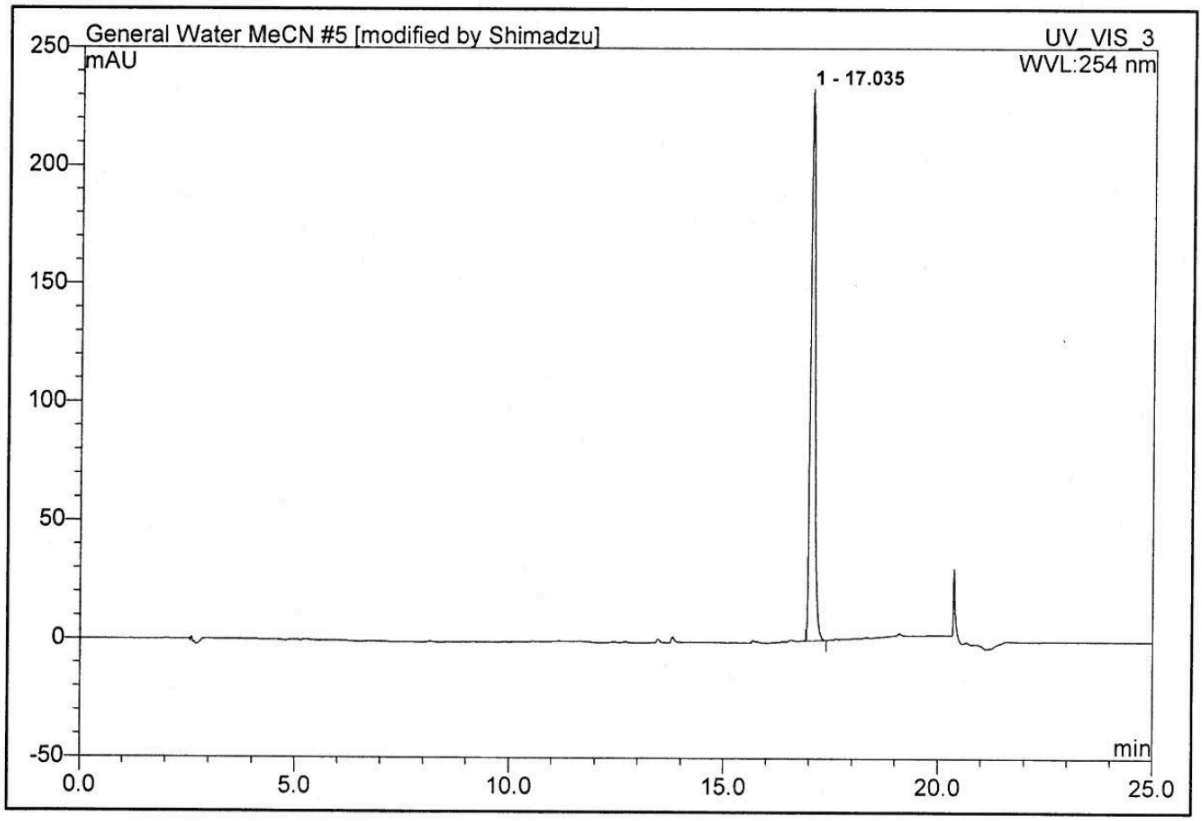


No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	16.52	n.a.	389.805	54.383	100.00	n.a.	BMB
Total:			389.805	54.383	100.00	0.000	

5 RM25

Compound 5d

Sample Name:	**RM25**	*Injection Volume:*	**20.0**
Vial Number:	**1_3**	*Channel:*	**UV_VIS_3**
Sample Type:	**unknown**	*Wavelength:*	**254**
Control Program:	**MeCN 25min gradient**	*Bandwidth:*	**8**
Quantif. Method:	**General Method**	*Dilution Factor:*	**1.0000**
Recording Time:	**24/2/2017 12:39**	*Sample Weight:*	**1.0000**
Run Time (min):	**25.00**	*Sample Amount:*	**1.0000**



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	17.03	n.a.	232.935	27.980	100.00	n.a.	BMB
Total:			232.935	27.980	100.00	0.000	

IV. Mass spec data

ROSHNI MALDE
1224846

protected Me ester ProTide 8a

Elemental Composition Report **Roshni Malde RM27** Compound 5a

Single Mass Analysis

Tolerance = 2.0 PPM / DBE: min = -1.5, max = 1000.0

Element prediction: Off

Monoisotopic Mass, Even Electron Ions

416 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)

Elements Used:

C: 0-100 H: 0-100 N: 0-4 O: 0-7 Na: 0-1 Si: 1-1 P: 1-1

Minimum:			-1.5		
Maximum:	5.0	2.0	1000.0		
Mass	Calc. Mass	mDa	PPM	DBE	Formula
480.1953	480.1947	0.6	**1.2**	5.5	**C21 H36 N O6 Na Si P**

ROSHNI MALDE
1224846

protected Bn ester ProTide 8b

Elemental Composition Report **Roshni Malde RM29** Compound 5b

Single Mass Analysis

Tolerance = 2.0 PPM / DBE: min = -1.5, max = 1000.0

Element prediction: Off

Monoisotopic Mass, Even Electron Ions

489 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)

Elements Used:

C: 0-100 H: 0-100 N: 0-4 O: 0-7 P: 1-1 Si: 1-1 Na: 0-1

Minimum:			-1.5		
Maximum:	5.0	2.0	1000.0		
Mass	Calc. Mass	mDa	PPM	DBE	Formula
556.2267	556.2260	0.7	**1.3**	9.5	**C27 H40 N O6 P Si Na**

ROSHNI MALDE
1224846

Protected ^{i}Pr ester ProTide 8c

Elemental Composition Report **Roshni Malde RM20**

Compound 5c

Single Mass Analysis

Tolerance = 2.0 PPM / DBE: min = -1.5, max = 1000.0

Element prediction: Off

Monoisotopic Mass, Even Electron Ions

444 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)

Elements Used:

C: 0-100 H: 0-100 N: 0-4 O: 0-7 Na: 0-1 P: 1-1 Si: 1-1

Minimum:			-1.5		
Maximum:	5.0	2.0	1000.0		
Mass	Calc. Mass	mDa	PPM	DBE	Formula
508.2259	508.2260	-0.1	**-0.2**	5.5	**C23 H40 N O6 Na P Si**

ROSHNI MALDE
1224846

Protected ^{t}Bu ester ProTide 8d

Elemental Composition Report **Roshni Malde RM25**

Compound 5d

Single Mass Analysis

Tolerance = 2.0 PPM / DBE: min = -1.5, max = 1000.0

Element prediction: Off

Monoisotopic Mass, Even Electron Ions

460 formula(e) evaluated with 1 results within limits (all results (up to 1000) for each mass)

Elements Used:

C: 0-100 H: 0-100 N: 0-4 O: 0-7 Na: 0-1 Si: 1-1 P: 1-1

Minimum:			-1.5		
Maximum:	5.0	2.0	1000.0		
Mass	Calc. Mass	mDa	PPM	DBE	Formula
522.2423	522.2417	0.6	**1.1**	5.5	**C24 H42 N O6 Na Si P**